Multiple sclerosis, remyelination and the role of fibronectin

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Chapter 1

Glial cells in health and multiple sclerosis

ABSTRACT

Cells constitute every organ in every organism. As such, cells are principal players in any disease. Hence, rational attempts to ameliorate disease will benefit from a thorough understanding of the cells involved and their specific functions. This thesis focuses on multiple sclerosis (MS), a disease of the central nervous system (CNS) with a central involvement of glial cells. Glial cells, a term referring to all CNS cells that are not neurons, comprise half the volume of the CNS and outnumber neurons by a factor of five to ten (Stevens 2003). Major glial cell types are oligodendrocytes, astrocytes and microglia, all of which play a role in MS pathology. In MS, myelin produced by oligodendrocytes is damaged and its biosynthesis is impaired, and a variety of functions from astrocytes and microglia change. In accordance with the concept that the development of rational therapeutic strategies requires basic molecular insights, first a brief overview of our current understanding of what comprises the myelin sheath will be given, followed by a short introduction to the glial cells mentioned. Then, contemporary insights into MS pathology are summarized, as well as the therapeutic options at hand. Finally, the current status of therapies that interfere with glial cell pathology in MS are discussed, in particular strategies aimed at the regeneration of myelin (remyelination).

THE MYELIN SHEATH COMPOSITION: A BRIEF OVERVIEW

Node, internode, paranode and juxtaparanode

Myelin is the extended plasma membrane from the oligodendrocyte, albeit with a unique composition of myelin-specific proteins and lipids. In multiple layers, myelin compactly wraps around an axon, the nerve fiber projected from a neuron (Fig. 1). One oligodendrocyte can provide myelin for up to sixty axons (de Monasterio-Schrader et al. 2012), which marks an important difference with Schwann cells from the peripheral nervous system (PNS), that wrap myelin only around one axon each. In addition, one CNS axon is usually ensheathed by myelin from more than one oligodendrocyte. However, not all oligodendrocytes make myelin and not all CNS axons are wrapped in myelin, although in complex, mammalian brains, the vast majority is (Wang et al. 2008). Along the axon, myelin sheaths and short, bare axon segments alternate. The myelin sheath is called the internode in this context, and the short stretches of bare axon are known as the nodes of Ranvier, which contain a high density of sodium channels. The internodal myelin sheath is tightly wrapped and compact, except for the innermost (periaxonal or adaxonal) and outer (abaxonal) loops, where myelin is non-compact. These differences in myelin tightness within the internode allow for the discrimination of the paranode, which refers to the inner, non-compact myelin layer with its underlying axon, and the juxtaparanode, comprising the 10 to 15 nanometer of myelin internode between the paranode on one side and the internode on the other (Baumann, Pham-Dinh 2001, Aggarwal, Yurlova & Simons 2011, Soldan, Pirko 2012) (Fig. 1).

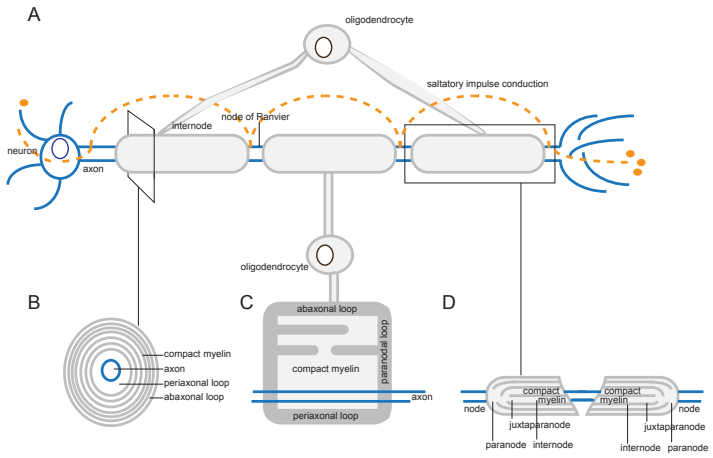

Figure. 1. Overview of the myelin sheath composition. A. Oligodendrocytes extend multiple processes to enwrap the axon from the neuron (blue) with a compact myelin sheath (pale grey; internode), leaving only small stretches of axon bare (node of Ranvier). This allows for saltatory conduction of nerve impulses (orange, dashed line), resulting in neurotransmitter release (orange circles). B. Cross section of the myelin sheath around the axon, showing compact myelin and the periaxonal and abaxonal loops. C. Unwrapped myelin sheath, revealing that myelin consists of compacted plasma membranes, with non-compacted cytoplasmic channels creating the abaxonal, paranodal and periaxonal loops. D. Closer look at compact myelin internode with the adjacent juxtaparanode, the non-compacted paranode, and the axonal node of Ranvier. Elements of the figure were adapted from (Aggarwal, Yurlova & Simons 2011, Soldan, Pirko 2012).

A polarized membrane with an extensive communication network

The myelin sheath has several striking features. First, the myelin membrane is uniquely polarized. Correct folding of the myelin membrane into the complex myelin structure described above requires a distinction between the plasma and myelin membranes, and further between the compact and non-compact regions of myelin (subpolarization). Although exact mechanisms for how the oligodendrocyte discriminates these membrane domains are still under investigation, the extraordinary molecular composition of the myelin sheath is considered pivotal (Baron, Hoekstra 2010, Simons, Snaidero & Aggarwal 2012). The lipid content of myelin is unusually high, with the main lipids being cholesterol, phospholipids, galactosylceramide (GalC) and its sulfated equivalent sulfogalactosylceramide or sulfatide (SGalC). The major proteins in the myelin sheath are myelin basic protein (MBP), particularly isoforms lacking exon II, and proteolipid protein (PLP) together with its isoform DM20. Other important proteins include myelin and lymphocyte protein (MAL), myelin oligodendrocyte glycoprotein (MOG), 'myelin associated glycoprotein (MAG), 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) and neurofascin-155 (NF-155). The myelin lipids and proteins tend to cluster at specific sites on the myelin sheath, enabling polarization and proper folding of the myelin sheath (which will be further discussed below). A second important feature of the myelin sheath is its surrounding communication network. The oligodendrocyte nucleus communicates with its own myelin internodes via channel-like tubes that originate from non-compact myelin sites and may run through the entire internode (Soldan, Pirko 2012). These tubes contain organelles, such as the endoplasmatic reticulum (ER), and vesicles, all of which may play a role in biosynthetic maintenance of the myelin sheath (de Monasterio-Schrader et al. 2012, Perkins et al. 2008). Furthermore, oligodendrocytes are often linked to other oligodendrocytes and to different CNS cells, such as astrocytes, among others via gap junctions (Aggarwal, Yurlova & Simons 2011, Kamasawa et al. 2005, Maglione et al. 2010), and therefore, an extensive network of communicating glia appears around the axon. Finally, oligodendrocytes express receptors for neurotransmitters, such as the NMDA-receptor for glutamate (Karadottir et al. 2005). Signaling via this receptor plays a role in the transfer of exosomes between neurons and oligodendrocytes (Fruhbeis et al. 2013). Further, neurotransmitter release from neurons may also evoke electrical currents in oligodendrocytes, although this is still under investigation (Karadottir, Attwell 2007).

WHY ARE OLIGODENDROCYTES AND MYELIN USEFUL?

Insulation of axons by myelin enables fast nerve impulse conduction, possibly saving energy

The first benefit of the myelin sheath is the insulation of the axon that it provides. Nerve impulses, or action potentials, result from changes in the axon membrane potential (depolarization) by an influx of sodium through sodium channels. Because 99.5% of the axon is insulated by myelin, depolarization needs to occur only at the remaining 0.5% of the surface area, i.e. at the nodes of Ranvier (Fig. 1). Hence, the action potential travels over the myelin sheath from node to node (of Ranvier), allowing for fast, saltatory nerve impulse conduction (Fig. 1). Without myelin, the axon

would have to tremendously increase its size in order to conduct nerve impulses as fast as with myelin. Also, depolarization exclusively at the node of Raniver means that myelinated axons need to restore ion gradients over a much smaller proportion of their membranes than unmyelinated axons. In this way, myelin cuts down on ATP requirements, because restoration of homeostatic ion gradients is largely driven by the ATP-dependent Na^+,K^+-ATPase pump (Nave 2010a). Indeed, in Long-Evans shaker rats, which bear an autosomal recessive mutation in MBP and therefore display gradual loss of myelin sheath integrity (Delaney et al. 1995), affected axons were found to contain more mitochondria (Smith, Cooksey & Duncan 2013). However, myelinated brain regions (white matter) use as much energy as areas that contain less myelin (grey matter), and therefore it has also been proposed that although myelin allows for rapid impulse conduction, it does not necessarily decrease overall energy demands of the CNS (Harris, Attwell 2012). Nonetheless, most scientists agree that myelin in one or both of these ways enabled evolution of large, yet fast and relatively energy-sparing nervous systems and, therefore, enabled better muscle control and more complex behavior. As such, myelin has likely been a critical factor in vertebrate evolution (Nave 2010a, Harris, Attwell 2012, Zalc, Goujet & Colman 2008).

Myelin may preserve axons

As a third benefit, oligodendrocytes may be required for the preservation of axons (Nave 2010a, Nave 2010b). For example, oligodendrocytes provide trophic support to axons via the delivery of pyruvate or lactate through monocarboxylic acid transporters (MCT)-1 and -2. This supplies the axons with a ready-to-use source of energy, and saves axons from performing glycolysis (Funfschilling et al. 2012, Lee et al. 2012b). In addition, adult oligodendrocytes express neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and insulin-like growth factor-1 (IGF-1) (Smith, Cooksey & Duncan 2013). Furthermore, oligodendrocytes may stabilize the axonal cytoskeleton and facilitate transport of axonal organelles (reviewed in: (Franklin et al. 2012b)).

Finally, the importance of myelin and oligodendrocytes is evident from diseases that primarily affect these cells, such as genetic leukodystrophies. A well-known example is Pelizaeus-Merzbacher disease, resulting from a recessive, X-linked mutation in the PLP gene. The type of mutation varies and so does the disease phenotype, but important clinical signs include nystagmus and hypotonia that usually progress to gait disturbance and spastic paraplegia. These symptoms generally develop in young children, signifying that oligodendrocyte progenitor cell (OPC) development, which occurs prenatally, is normal, but postnatal myelin folding to a compact wrap is severely disturbed (Garbern 2007). What, then, is required for normal oligodendrocyte development and myelination?

THE MYELIN MAKERS AND MORE: OLIGODENDROCYTE PROGENITOR CELLS AND OLIGODENDROCYTES

Specific transcription factors mediate the generation of oligodendrocyte progenitor cells from neural stem cells

During embryonic development of the vertebrate CNS, when many neurons have already been developed, OPCs are derived from neural stem cells in the neural tube. Different cell types develop from the same neuroepithelium, because the neural tube is patterned into distinct domains by regional gradients of specific morphogens. These morphogens are bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wnt for the roof plate or dorsal zone, and sonic hedgehog (Shh) for the floor plate or ventricular zone (Fig. 2). In the patterned domains, cells start to express

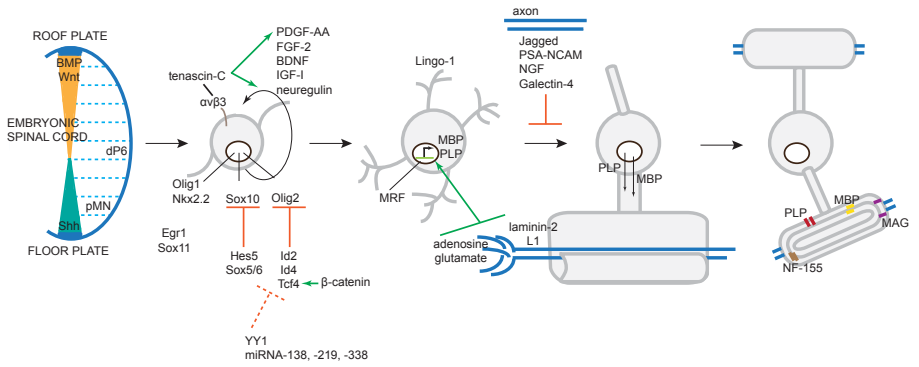

Figure 2. Oligodendrocyte progenitor cell development and lineage progression towards myelinating oligodendrocytes. The embryonic spinal cord is patterned into distinct domains by regional gradients of the morphogens bone morphogenic protein (BMP), Wnt and sonic hedgehog (Shh). Neural progenitor cells from the dP6 and pMN domains develop into oligodendrocyte progenitor cells. Oligodendrocyte progenitor cells are repressed in their undifferentiated state through a complex inhibitory transcription factor network, of which several key players are shown. Olig2 expression is prevented by Inhibitor of DNA-binding-2 (Id2), Id4 and transcription factor 4 (Tcf4), for which β-catenin is an important stimulant. Further, Sox10 is inhibited by Hes5 and Sox5/6, with additional important transcription factors being Olig1, Nkx2.2, Egr1 and Sox11. Oligodendrocyte progenitor cells proliferate under the influence of growth factors, including platelet-derived growth factor-AA (PDGF-AA), fibroblast growth factor-2 (FGF-2), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-I (IGF-I) and neuregulin. These growth factors signal together with extracellular matrix molecules, such as tenascin-C, which binds to the integrin receptor αvβ3. On removal of signaling from the inhibitory transcription factor network, mediated among others by YingYang-1 (YY1) and the micro RNAs (miRNA) -138, -219, and -318, oligodendrocyte progenitor cells develop into pre-myelinating oligodendrocytes. Transcription of the important myelin proteins myelin basic protein (MBP) and proteolipid protein (PLP) is initiated. To prevent premature myelination, oligodendrocytes express Lingo-1 and axons (blue) synthesize several factors, including Jagged, polysialylated-neural cell adhesion molecule (PSA-NCAM), nerve growth factor (NGF) and galectin-4. Conversely, presumably at the correct time, axons also promote myelination from signaling via adenosine and glutamate, as well as the axonal adhesion molecules laminin-2 and L1. Correct folding of the oligodendrocyte plasma membrane into the compacted myelin sheath requires, among others, polarized transport of PLP and MBP. The compacted structure is only maintained if myelin gene regulatory factor (MRF) is continuously expressed from the post-mitotic stage, and all the elements of the sheath are properly localized, shown here for neurofascin-155 (NF-155; brown) in the paranode, PLP (red) and MBP (yellow) at distinct sites in the internode, and myelin-associated protein (MAG) in the periaxonal site. Elements of the figure were adapted from (Emery 2010, Fancy et al. 2011).

specific transcription factors relevant for the development towards their eventual cell fate (Rowitch, Kriegstein 2010). The majority of OPCs originate from neural progenitor cells in the motoneuron (pMN) domain of the ventricular zone. Specifically, on Shh signaling here, the transcription factors Nkx6 and Olig2 are transcribed, initiating the differentiation of ventral neural progenitor cells into motor neurons and OPCs (Briscoe et al. 2000, Zhou, Anderson 2002). In addition, a smaller proportion of OPCs originate from neural progenitors in the dorsal neuroeptihelium (dP6) (Cai et al. 2005, Vallstedt, Klos & Ericson 2005, Fogarty, Richardson & Kessaris 2005) (Fig. 2). OPCs can be recognized by the expression of transcription factors (as discussed below), platelet-derived growth factor receptor alpha (PDGFR-α) and the proteoglycan NG2 (Rivers et al. 2008, Zhu, Bergles & Nishiyama 2008, Zhu, Hill & Nishiyama 2008), although PDGFR-α is expressed by many other cell types outside the CNS, and NG2 also marks immature Schwann cells, pericytes and muscle progenitor cells (Trotter, Karram & Nishiyama 2010). OPCs display some lineage plasticity, in that they can also differentiate into neurons (Rivers et al. 2008), astrocytes (Tatsumi et al. 2008) and Schwann cells (Zawadzka et al. 2010), but the vast majority of OPCs commits to differentiate into oligodendrocytes (reviewed in: (Richardson et al. 2011)).

Migration and proliferation of oligodendrocyte progenitor cells is promoted by a variety of signaling pathways

Before differentiating into myelin-producing oligodendrocytes, OPCs proliferate and migrate throughout the developing CNS. To this end, motility of OPCs is increased, most notably by PDGF-AA from astrocytes (Fruttiger et al. 1999, Baron, Shattil & ffrench-Constant 2002b) and FGF-2 (McKinnon et al. 1990) that activates several mitogen-activated protein kinase (MAPK) pathways downstream (Baron et al. 2000). Simultaneously, OPCs proliferate to increase their numbers, which is guided by a variety of extracellular signals. Among these signals are growth factors, such as BDNF signaling to tyrosine kinase receptor-B (trkB) (Van't Veer et al. 2009, Wong et al. 2013), PDGF-AA (Fruttiger et al. 1999), neuregulin (Canoll et al. 1996), IGF-1 (McMorris, Dubois-Dalcq 1988) and FGF-2 (McKinnon et al. 1991), while IGF-1 and FGF-2 can also synergize (Jiang, Frederick & Wood 2001) (Fig. 2). Cellular sources of these growth factors are OPCs themselves, but also, for example, astrocytes for PDGF-AA and neurons for neuregulin (Fruttiger et al. 1999, Canoll et al. 1996). In addition to these long-range signals, some extracellular matrix (ECM) proteins act as local promoters of OPC proliferation, for instance tenascin-C (Garcion, Faissner & ffrench-Constant 2001). OPCs recognize ECM proteins among others via a variety of integrin receptors, namely αvβ3, αvβ8, α6β1, αvβ5 and αvβ1 (Baron, Colognato & ffrench-Constant 2005, O'Meara, Michalski & Kothary 2011). Binding of ECM ligands to integrin receptors on OPCs provides a means of integrating long-range cues from growth factors with short-range cues from the ECM. For example, proliferation of OPCs on PDGF-AA benefits from binding of ligands to αvβ3, with binding partners being the local ECM proteins fibronectin and vitronectin in addition to PDGF-AA itself (Baron, Shattil & ffrench-Constant 2002a). Further, tenascin-C, a ligand for αvβ3, amplifies responsiveness of OPCs to PDGF-AA (Garwood et al. 2004)

(Fig. 2). Migration of OPCs throughout the CNS occurs in parallel to proliferation and is stimulated to some extent by the same factors. Accordingly, PDGF-AA also acts as a chemoattractant (Zhang et al. 2004), similar to semaphorins of class 3 and netrin-1 (Spassky et al. 2002). Additional signals to enhance proliferation of OPCs include neuregulin-1 (Ortega et al. 2012) and ECM proteins, such as anosmin-1 (Bribian et al. 2008) and laminin-2 (Frost et al. 1996). To promote OPC proliferation and migration, integrin activation results, among others, in cytoskeletal remodeling downstream, for which the phospoinositide-3-kinase (PI3K)/Akt and Ras/MAPK pathways are important. Integrin receptors also interact with other receptors, such as the epidermal growth factor (ErbB) receptor (O'Meara, Michalski & Kothary 2011) and PDGFR-α, which further enhances proliferation and migration (Baron, Colognato & ffrench-Constant 2005, Baron, Shattil & ffrench-Constant 2002a, Baron et al. 2003). Finally, electrical activity from axons is an important regulator for proliferation and migration, as well as throughout the myelination process. Early evidence for the necessity of action potentials for myelination was provided by studies on newborn mice that were reared in the dark. Without light, and hence without much electrical activity in their optic nerves, these mice displayed a substantial decrease in myelinated optic nerve fibers (Gyllensten, Malmfors 1963). Moreover, early opening of rabbit eyes facilitates early myelination (Tauber, Waehneldt & Neuhoff 1980) (reviewed in (Coman et al. 2005)). Proliferation of OPCs is stimulated by electrical activity via the release of PDGF-AA (Barres, Raff 1993, Gallo et al. 1996). Similarly, migration is promoted by reducing binding of αv integrin on OPCs to the ECM (Gudz, Komuro & Macklin 2006). Additional roles for electrical signals at later stages in the myelination process will be discussed below.

Differentiation of oligodendrocyte progenitor cells according to the derepression model

Maturation of OPCs involves their progression through different development stages from immature, premyelinating oligodendrocytes towards mature, myelinating oligodendrocytes (Fig. 2), with myelination taking place predominantly after birth. Differentiation of OPCs into oligodendrocytes requires appropriate timing, so that eventual myelination is initiated when both the OPC and the axon are ready. Among others, this careful coordination in time and space avoids myelin assembly from occurring inside the OPC or in processes that are not within close reach of axons (Simons, Trotter 2007). In fact, myelination is such a neatly timed process that assessing the extent of myelination in a human fetus allows for a precise determination of its age (Friede 1973, Wang et al. 1998), and the time window of opportunity for developmental myelination to take place is considered extremely narrow (Czopka, ffrench-Constant & Lyons 2013). The intrinsic regulation of OPC differentiation is proposed to occur according to the 'derepression model' of myelination (Casaccia-Bonnefil, Liu 2003, Wegner 2008, Emery 2010). In this model, OPCs are repressed in their undifferentiated state by the continuous expression of transcription factors that inhibit myelin gene expression, and this repression is mediated by extracellular signals. When the environment is permissive for oligodendrocyte differentiation, downregulation of the inhibitory transcription factors occurs in OPCs, which enables transcription of myelin genes. Important transcription factors that stimulate

myelin gene expression, including the transcription of PLP and MBP, are Olig2 (Zhou, Anderson 2002) and Sox10 (Wegner 2008, Stolt et al. 2003). Hence, to maintain OPCs in their undifferentiated state, repression of Olig2 and Sox10 is required. The inhibitory transcription factors that repress Olig2 comprise Inhibitor of DNA-binding-2 (Id2), Id4 and transcription factor 4 (Tcf4) (Wegner 2008, Kondo, Raff 2000, Samanta, Kessler 2004, Marin-Husstege et al. 2006, He et al. 2007). At this stage, OPCs express the G-protein coupled receptor-17 (GPR-17), which may enforce Id2/Id4 activity (Chen et al. 2009). Furthermore, an important regulator of Tcf4 expression in OPCs is stabilized β-catenin resulting from Wnt signaling (Fancy et al. 2009). Myelin gene transcription from Sox10 activity is repressed by the transcription factors Hes5 (Liu et al. 2006) and Sox5/6 (Stolt et al. 2006) (reviewed in (Wegner 2008)). On maturation of OPCs, the expression of Id4 and Tcf4 is eventually inhibited by Ying Yang-1 (YY1) via chromatin remodeling through histone deacetylase-1 (HDAC-1) (Wegner 2008, He et al. 2007). Other transcription factors that are essential for myelination include Olig1 (Xin et al. 2005) and Nkx2.2 (Qi et al. 2001), and additional important inhibitory transcription factors are Egr1 and Sox11 (Swiss et al. 2011).

At the controls of this inhibitory transcription factor network (or: 'transcriptome') sits the OPC together with its environment. Internal control of maturation from OPC to oligodendrocyte is evident from primary oligodendrocyte cultures without any other cells – most notably axons – in which OPCs still mature towards oligodendrocytes, albeit with flat myelin sheets rather than sheaths (Dubois-Dalcq et al. 1986). However, *in vivo*, the environment interferes with rapid lineage progression, presumably to ensure that myelination is properly timed. In particular axons communicate with OPCs at different steps of the myelination process. First, axonal signals are important to inhibit premature OPC differentiation. Inhibitory signaling molecules from axons include Jagged1 to the Notch1 receptor on OPCs (Wang et al. 1998), polysialylated-neural cell adhesion molecule (PSA-NCAM) (Charles et al. 2000), nerve growth factor (NGF) to tyrosine kinase receptor-a (TrkA) on OPCs (Chan et al. 2004) and galectin-4 (Stancic et al. 2012). Also, the transmembrane protein Lingo-1 in differentiating OPCs inhibits premature myelination (Mi et al. 2005) (Fig. 2). Secondly, axonal signals participate in target-dependent survival. This process, also named 'oligodendrocyte pruning' (Piaton, Gould & Lubetzki 2010), describes how, during CNS development, a substantial proportion of OPCs undergoes apoptosis, because OPCs are physiologically generated in excess relative to final oligodendrocyte numbers in the mature CNS (Barres et al. 1992). The majority of surviving OPCs are those that establish contacts with axons, and one of the survival signals involved is laminin-2 from axons, which binds to the α6β1 integrin receptor on OPCs (Frost et al. 1999). Thirdly, axonal molecules promote differentiation of selected OPCs into oligodendrocytes before the actual myelination process commences. Axonal molecules that stimulate differentiation of OPCs include laminin-2 signaling to α-dystroglycan and α6β1 integrin, with the latter facilitating MAPK activation on neuregulin-1 (Colognato et al. 2002, Colognato et al. 2007). Laminin also acts via Fyn, focal adhesion kinases and Rho GTPases to change actin dynamics and initiate OPC differentiation (Hoshina et al. 2007). Although laminin is not the single essential factor in myelination, its relative

significance is clear from regional hypomyelination that is observed in *dy/dy* mice, which are largely deficient in laminin-α2 (Chun et al. 2003). In fact, based on the complex molecular mechanisms involved in CNS myelination, it is considered unlikely that this process is under control of a single essential molecular element at all. This marks a difference with PNS myelination, where Schwann cell-mediated myelination is largely controlled by axonal growth factor neuregulin-1 type III signaling to ErbB receptors (Michailov et al. 2004, Taveggia et al. 2005). In addition to axonal molecules, electrical activity from axons also promotes differentiation of OPCs (Demerens et al. 1996). This may be mediated by neurotransmitters released from axon potentials, such as adenosine signals to purinergic receptors on OPCs (Stevens et al. 2002). Furthermore, some OPCs respond to glutamate with membrane depolarization, resulting from ion travel through voltage-gated sodium and potassium channels (Karadottir et al. 2008), although it is still unclear how this affects their lineage progression and myelination (reviewed in (Emery 2010)). Alongside stimulatory axonal factors, differentiation of OPCs also requires downregulation of transcription factors that inhibit myelin gene expression. Important mediators to downregulate these transcription factors are microRNAs, a type of small non-coding RNAs that inhibit gene expression after transcription by preventing translation or by degrading mRNA. During differentiation of OPCs, microRNAs are induced, in particular microRNA-138, -219 and -338, that downregulate Sox6, Hes5 and PDGFR-α among others, thereby exiting OPCs from the proliferative cell cycle (Dugas et al. 2010, Zhao et al. 2010). Further, post-mitotic oligodendrocytes express the transcription factor myelin-gene regulatory factor (MRF), which is essential for myelination and myelin maintenance (Emery et al. 2009, Koenning et al. 2012).

Biosynthesis and compaction of the myelin sheath involves axonal signals as well as site-specific clustering of myelin proteins and lipids

Once OPCs are differentiated into oligodendrocytes, they face the task of extending their plasma membrane to the axons and produce myelin. This is again a complex, multi-step program, requiring polarization (Baron, Hoekstra 2010, Simons, Trajkovic 2006). First, the oligodendrocyte plasma membrane needs to recognize and adhere to the correct axon, avoiding interactions with dendrites (Lubetzki et al. 1993). Then, myelin proteins and lipids are synthesized and transported in a polarized manner to the plasma membrane extensions or processes, followed by biogenesis of the sheath. Finally, the sheath is wrapped around the axon in multiple layers, before compaction to its mature form occurs. Axons play an important role in the coordination of myelination at this stage. Biochemically, the axonal adhesion molecule L1 and laminin-2 signal to the α6β1 receptor, expressed on the surface of oligodendrocytes, to activate Fyn kinase, which promotes MBP mRNA synthesis and transport to the correct site in the outgrowing oligodendrocyte processes (White et al. 2008, Laursen, Chan & ffrench-Constant 2009). Also, laminin-2 signals via the dystroglycan receptor to promote myelin membrane formation (Colognato et al. 2007). In addition, action potentials may play a role. For example, the neurotransmitter glutamate signals to NMDA and metabotropic glutamate receptors on oligodendrocytes to activate Fyn kinase which, as indicated, promotes MBP

synthesis (Wake, Lee & Fields 2011) (Fig. 2). Finally, by an indirect mechanism, the release of ATP from action potentials stimulates astrocytes to synthesize leukemia inhibitory factor (LIF), which also promotes myelination by oligodendrocytes (Ishibashi et al. 2006).

In the final steps of myelination, the myelin sheath is properly folded around the axon and compacted into its definite form. In order to achieve this, site-specific clustering of myelin proteins and lipids is required. This involves, among others, polarized transport and proper positioning of MBP and PLP to distinct sites in the internode, that of NF-155 to the paranode, and MAG to the periaxonal site (Simons, Trajkovic 2006) (Fig. 2). In addition, glycolipid-enriched microdomains, termed 'lipid rafts', are generated, which also contribute to correct positioning of myelin molecules at axon-myelin sheath interaction sites, and hence to myelin assembly (reviewed in: (Gielen et al. 2006)). MBP, which exists in different isoforms that may have different functions (de Vries et al. 1997, Harauz, Boggs 2013), is transported from the oligodendrocyte nucleus to the myelin sheath in the form of mRNA, packed in granules, which are only translated into MBP in the sheath, presumably to prevent premature membrane clustering. Conversely, after protein translation at the ER, the transport of PLP occurs in vesicles, via the Golgi to the plasma membrane and subsequently to the myelin sheath in a process known as transcytosis (Baron, Hoekstra 2010, Trajkovic et al. 2006). The coordination of correct and polarized transport from these and other proteins and lipids is complex and incompletely understood, although they are likely mediated by axonal cues (Simons, Trajkovic 2006, Trajkovic et al. 2006), with important roles for axonal adhesion factors and secreted soluble factors (reviewed in: (Baron, Hoekstra 2010, Simons, Trotter 2007, Maier, Hoekstra & Baron 2008). However, the observation that oligodendrocytes myelinate synthetic nanofibers (Lee et al. 2012a) indicates that axonal signals may also be largely non-specific and perhaps more important for correct timing of myelination. Finally, important players in the myelin-related trafficking machinery are likely Rab proteins and soluble NSF attachment protein receptor (SNARE) proteins, including distinct vesicle-associated membrane proteins (VAMPs), acting as v-SNARES, and syntaxins, as part of the t-SNARES at the recipient target membrane (Simons, Trajkovic 2006, Feldmann et al. 2009).

Oligodendrocyte progenitor cells in the adult central nervous system

During adulthood, OPCs remain present throughout the CNS (Pringle et al. 1992, Dawson et al. 2003). An important source of OPCs in the adult are neural stem cells from specific niches, such as the subventricular zone (Menn et al. 2006a), and signals to induce OPC formation from these neural stem cells include Wnt (Ortega et al. 2013) and those repressing sirtuin 1 (SIRT1) (Rafalski et al. 2013). The purposes of OPCs in the healthy, adult CNS have not yet been fully elucidated. Yet, it is likely that OPCs differentiate and continue to generate myelin during adulthood (Young et al. 2013), and in this way may replace 'old' oligodendrocytes and add to existing myelin (reviewed in (Richardson et al. 2011)). Indeed, myelin appears to be a dynamic structure in the adult, because myelination continues until late in life (Fields 2008). Interestingly, myelination may even increase in specific regions on practicing of particular skills, for example juggling (Scholz et al. 2009) or piano playing

(Bengtsson et al. 2005). For this myelin plasticity, myelin is hypothesized to play an important role in cognitive processes, such as learning, and cognitive diseases, among others depression and schizophrenia (reviewed in (Fields 2008)). OPCs are also pivotal in myelin regeneration after injury, which will be discussed further below.

ASTROCYTES

Phenotypic properties of astrocytes

Astrocytes are the most abundant cells of the CNS. Development of astrocytes starts in the embryonic, patterned neural tube, where astrocytes derive from neural progenitor cells in the ventricular zone. Important transcription factors to promote development of neural progenitor cells into the astrocyte lineage are nuclear factor-IA (NFIA) (Deneen et al. 2006) and Sox9 (Kang et al. 2012). Subsequently, astrocytes migrate throughout the developing CNS in strict accordance to their original embryonic site (Tsai et al. 2012). In the adult, astrocytes do not normally proliferate, except for after injury to the CNS (Buffo et al. 2008). On invasive injuries, such as stab wounding, astrocytes can acquire neural stem cell properties again via Shh signals (Sirko et al. 2013). Astrocyte morphologies differ per brain region, and on injury, astrocytes from different regions cannot fully replace each other functionally (Tsai et al. 2012). In the grey matter, astrocytes are protoplasmic with ramified branches that connect to synapses and blood vessels. In the white matter, astrocytes are fibrous (or fibrillary) and have longer and thinner processes that contact nodes of Ranvier and blood vessels. Further, additional astrocyte phenotypes can be recognized in specific brain locations, such as Bergmann glia in the cerebellum and Müller glia in the retina (reviewed in: (Barres 2008, Sofroniew, Vinters 2010)). Astrocytes express glial fibrillary acidic protein (GFAP) and aldehyde dehydrogenase 1 family member L1 (Aldh1L1) (Lovatt et al. 2007, Cahoy et al. 2008). Mutations in the GFAP gene are associated with Alexander's disease that causes clinical symptoms ranging from convulsions and mental retardation to bulbar signs, muscle weakness and hyperreflexia. How GFAP and, by extension, astrocyte dysfunction mediates these symptoms is unknown (Yoshida, Nakagawa 2012), and in fact, functions of astrocytes in the healthy brain are still far from being completely understood (Barres 2008)

Functions of astrocytes

Thus far, astrocytes have mainly been considered support cells for the blood-brain barrier (BBB) and neurons. The BBB consists of closely linked endothelial cells, sealed via tight junctions, and connected to a basal lamina of extracellular matrix proteins, including laminin and fibronectin, and to pericytes and astrocytes (Lampron, Elali & Rivest 2013). The BBB protects the parenchyma of the CNS against entry of potentially adverse molecules, whereas it facilitates the entry of nutrients via specific transporters. In addition, the BBB is closely associated with neurons to regulate a correct CNS ion balance. Ultimately, cerebral blood flow is largely controlled by neuronal activity (Koehler, Roman & Harder 2009). Astrocytes structurally support the BBB with their end-feet that are connected to the

capillaries, largely via integrin receptors (Koehler, Roman & Harder 2009). In addition, astrocytes play a role in regulating blood flow through the capillaries (Hawkins, Davis 2005), although molecular mechanisms behind this remain to be elucidated (Koehler, Roman & Harder 2009). Astrocytes also sustain neurons (Rowitch, Kriegstein 2010), and they may participate in neuronal signaling at the synapse in the concept of the 'tripartite synapse'. To modify synaptic transmission, astrocytes may release neurotransmitters (Araque et al. 1999), although there is controversy over whether astrocytes release glutamate (Barres 2008) or any other neurotransmitter (Agulhon et al. 2008) *in vivo*. However, astrocyte signaling at the synapse may also occur independent of neurotransmitters (Nedergaard, Verkhratsky 2012). Finally, astrocytes are mediators of the innate immune response in the brain (Ransohoff, Brown 2012), which will be discussed next.

ARCHITECTURE OF THE BRAIN IMMUNE SYSTEM: MICROGLIA AND MACROPHAGES

The central nervous system is relatively immune privileged

The immune defense system of the brain is unlike that of any other organ. The healthy brain parenchyma is largely depleted of adaptive immune cells, with T-cells predominantly residing in the cerebrospinal fluid (CSF). Cells with innate immune functions are present, primarily astrocytes and microglia, but also a minority of mast cells and macrophages are settled in the meninges and in perivascular spaces of parenchyma capillaries and arteries (Ransohoff, Engelhardt 2012). The brain is relatively 'immune privileged', meaning that delivery of immunogenic material directly to the brain parenchyma does not elicit a classical, adaptive immune response. Such a classical immune response would require antigen presentation to naïve T-cells (the afferent arm), and the subsequent entry of activated T-cells into the brain parenchyma, thereby resulting in local immune activity (the efferent arm). The immune privilege of the brain is principally considered to be mediated by unusual functioning of the afferent arm, in that CNS antigen presenting cells (APCs) cannot easily travel to lymph nodes to present antigens to naïve T-cells (Galea, Bechmann & Perry 2007). APCs in the CNS comprise the meningeal and perivascular macrophages, whereas microglia, although they express some major histocompatibility class (MHC) II molecules, have a limited capacity to present antigens (Ransohoff, Cardona 2010). Despite the limitations in APC travel to lymphoid organs, soluble antigens drain through the perivascular spaces to lymphatic vessels in the nasal submucosa (reviewed in: (Galea, Bechmann & Perry 2007)). Also, memory T-cells likely enter the CNS from the CSF by various trafficking mechanisms, including α4β1 integrin-mediated transmigration across the BBB (Ransohoff, Engelhardt 2012, Ousman, Kubes 2012). This implies that the brain is relatively immune privileged, but does not completely lack adaptive immunity.

Microglia are likely versatile cells with a function in brain immunity

Astrocytes and microglia mediate innate immune responses in the CNS. Astrocytes express innate immune receptors, such as toll-like receptor (TLR)-2 and TLR-3 (Bsibsi et al. 2002) and nucleotide-binding oligomerization domain receptor (NLR) proteins (Minkiewicz, de Rivero Vaccari & Keane

2013). On immunological activation, astrocytes express complement factors, interleukins and other cytokines and chemokines (reviewed in: (Ransohoff, Brown 2012)). Microglia originate from myeloid yolk sac progenitors, which colonize the CNS early in embryonic life, and they maintain their population by local proliferation (Ginhoux et al. 2010). Hence, microglia classify as haematopoietic cells, unlike macroglia (such as oligodendrocytes and astrocytes) and neurons (Ransohoff, Cardona 2010). Indeed, microglia share many properties with macrophages, and cannot be discriminated from macrophages by known biochemical markers. Microglia express all TLRs (van Noort, Bsibsi 2009, Lehnardt 2010), and on engagement of these receptors display innate immune activity similar to astrocytes, albeit generally more fulminantly (Ransohoff, Brown 2012). Furthermore, several microglia phenotypes can be distinguished in correspondence with the M1/M2 concept for macrophages (Durafourt et al. 2012). In this concept, macrophages and microglia can display a pro-inflammatory phenotype (M1), for instance if activated by interferon-γ (IFN-γ), to enhance antigen presentation properties and secrete pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-12, as well as reactive oxygen species. Conversely, an alternatively activated profile (M2) has been described as well, and was later subdivided into the M2a, M2b and M2c subtypes. In M2a and M2c, microglia and macrophages display anti-inflammatory and pro-regenerative features; for M2a including enhanced expression of arginase-1, the mannose receptor, IGF-1 and PDGF, and for M2c comprising IL-10 and TGF-β expression. The M2b phenotype is associated with the humoral response to bacteria, and thus involves macrophage and microglia secretion of TNF-α, Il-1β and IL-12 (Gordon 2003, Edwards et al. 2006, Martinez, Helming & Gordon 2009). In addition, intermediate activation types likely exist.

However, microglia maintain brain homeostasis also independent of immune activity. In the healthy brain, ramified microglia elongate and retract their processes unremittingly, likely to monitor the environment (Nimmerjahn, Kirchhoff & Helmchen 2005). Non-activated microglia promote survival and maturation of developing OPCs (Nicholas, Wing & Compston 2001). Similarly, ramified microglia contribute to the regulation of neuronal cell numbers in developmental and adult neurogenesis, partly independent of phagocytosis. Furthermore, microglia likely participate in neuronal circuit formation via direct interactions with neurons (reviewed in: (Wake et al. 2013)). Also, microglia may mediate myelin turnover via macropinocytosis of exosomes from oligodendrocytes (Fitzner et al. 2011). Finally, primary microglia dysfunction may partly explain the pathogenesis of Nasu-Hakola disease (also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy). This disease, which affects patients with recessively inheritable mutations in the DAP12 and TREM2 genes, is characterized by the insidious development of ossal pain from bone cysts and subsequent character changes and dementia from neurodegeneration. Because DAP12 and TREM2 are exclusively expressed in osteoclasts and myeloid cells, microglia are implied in Nasu-Hakola pathogenesis (Bianchin et al. 2004). In short, to regard microglia solely as brain resident macrophages, with a primary immune function, could well impose a limited view on these cells (Graeber 2010).

Having discussed roles of glial cells in health, we will now focus on their involvement in the CNS disease multiple sclerosis (MS).

MULTIPLE SCLEROSIS

Clinical symptoms of multiple sclerosis

MS is a CNS disease of diverse symptoms and progression. Patients, usually between the ages of 15 and 50 years at onset and predominantly females, typically suffer from sensory and motor symptoms, visual loss, gait disturbance, balance problems, vertigo and/or bladder problems. These symptoms develop from lesions at specific sites within the CNS. To make the diagnosis of MS therefore requires the demonstration of such lesions and of their dissemination in time and space according to the 2010 revisions of the McDonald criteria (Polman et al. 2011). The disease course of MS is variable and fairly unpredictable. A relapsing-remitting pattern (relapsing-remitting MS), in which symptom attacks and recovery periods alternate, is most common. A minority of patients suffers few attacks and recovers completely (benign MS). However, rather than recovery, patients may also experience steady decline of neurological functions between relapses from disease onset onwards (progressive relapsing MS), or deteriorate after an initial period of relapsing-remitting MS (secondary progressive MS). In addition, approximately 10% of MS patients do not experience recovery periods at all and deteriorate straight from the onset of the disease (primary progressive MS) (Weinshenker 1994, Compston, Coles 2008, Koch-Henriksen, Sorensen 2010). Eventually, disabilities accumulate in the majority of MS patients, usually within the course of several decades of time (Tremlett, Paty & Devonshire 2006). Therefore, the burden of MS is often considered as one of disability rather than increased mortality. MS patients are, however, likely also at a three-fold increased risk for premature death, most commonly due to respiratory disease (Hirst et al. 2008, Lalmohamed et al. 2012).

Pathological hallmarks of multiple sclerosis are versatile and involve neurons as well as glial cells

The ethiology of MS lesions is still incompletely understood, despite intense scientific research. Many factors have been suggested by inference. These include Eppstein-Barr virus infection (Tzartos et al. 2012, Serafini et al. 2013) and reduced vitamin D levels, but also cigarette smoking (reviewed in: (Ascherio, Munger & Lunemann 2012)), Further, susceptibility for MS is also associated with certain genetic architectures, especially with genes implied in (cell-mediated) immunity and human leukocyte antigen (HLA) composition for relapsing-remitting patients (International Multiple Sclerosis Genetics Consortium et al. 2011). MS is most prevalent in Europe, North America, Australia and New Zealand, where up to 0.1% of the population is affected (Koch-Henriksen, Sorensen 2010). However, whether there is a latitudinal gradient for MS incidence, which would suggest a trigger from shared environmental exposure, is currently debated (Koch-Henriksen, Sorensen 2010, Poser, Brinar 2007)

Several pathological hallmarks are apparent on examining an archetypal acute, active MS lesion

(or 'plaque') in relapsing-remitting MS patients. Loss of oligodendrocytes occurs with degeneration of myelin (demyelination) (Barnett, Prineas 2004). Possibly as a result of demyelination, axons are degraded, especially thin axons (Evangelou et al. 2001). Inflammation is evident from activated macrophages with ingested myelin sheath fragments, perivascular infiltrates containing T-cells, and a minority of B-cells (Lassmann, Bruck & Lucchinetti 2007, Lassmann 2011). Microglia also display an activated morphology (Lassmann, Bruck & Lucchinetti 2007) and microglia and infiltrated macrophages are of an intermediate activation status, possibly predominantly of the M1 phenotype (Vogel et al. 2013). Microglia and macrophages also express NADPH oxidase that may participate in oxidative tissue damage (Fischer et al. 2012). Autoantibodies from the complement system against axons or myelin are present in a subset of patients (Elliott et al. 2012), and some target the potassium channel Kir4.1, thereby likely damaging astrocytes and oligodendrocytes (Srivastava et al. 2012). To complete the inflammatory events, astrocytes become hypertrophic and possibly present antigens, because they may express MHC molecules (Zeinstra et al. 2000). Also, astrocytes likely become activated in other ways, for example by expressing an excess of the free radical precursor NADPH (Trapp et al. 1999) and nitric oxide (NO) (De Groot et al. 1997, Oleszak et al. 1998). A final pathological feature is the disruption of the BBB (Kirk et al. 2003), which is reflected in an enhanced accumulation of gadolinium in active MS lesions when visualized by magnetic resonance imaging (MRI). In progressive MS, lesions display largely similar pathological features, but the BBB is often intact and axonal injury becomes more pronounced (reviewed in: (Lassmann, van Horssen & Mahad 2012)). Active and chronic MS lesions can be further sub classified by differences in the density and distribution of inflammatory cells (Lassmann 2011). MS lesions occur throughout the CNS white matter, but also grey matter (reviewed in: (Geurts, Barkhof 2008)) with cortical lesions accumulating especially during progressive MS (Peterson et al. 2001). In cortical lesions, pathological events are similar to those in white matter lesions, notably also with respect to inflammatory features (Kutzelnigg et al. 2005, Lucchinetti et al. 2011), although inflammation may not be as abundant in cortical lesions later in the disease (Peterson et al. 2001).

Hypotheses to explain the pathogenesis of multiple sclerosis

Which pathological events cause MS lesion formation, and which are merely consequences of the disease process? The definite answer to this question is not yet clear, but several hypotheses are tested and refined. The autoimmune hypothesis broadly states that myelin-reactive T-cells invade the brain, possibly after switching to a migratory phenotype in the lung (Odoardi et al. 2012), where they elicit local immune responses and demyelination with secondary axonal degeneration (reviewed in: (Nylander, Hafler 2012)). Although the autoimmune hypothesis explains several aspects of MS, including the similarities to the experimental autoimmune encephalomyelitis (EAE) animal model as well as some of the therapeutic benefits of immunosuppressive agents, other features of MS are inconsistent with autoimmunity (reviewed in: (Stys et al. 2012)). Primary demyelination is apparent in at least a subset of MS patients (Lucchinetti et al. 2000), and hence,

in a second hypothesis, it is stated that oligodendrocyte pathology could initiate MS. However, whereas inducing oligodendrocyte death in experimental animal models provokes demyelination and activation of macrophages and microglia, this does not generate an immune response from T-cells, even under conditions favoring autoimmunity (Locatelli et al. 2012, Caprariello et al. 2012). Further, others explore whether MS could in effect be a neurodegenerative disorder (Trapp, Nave 2008). Still others investigate the possibility that multiple degenerative and immunological processes occur in parallel, with a central role for changes in copper-dependent regulation of NMDA receptors on myelin (Stys et al. 2012) and/or oxidative stress and mitochondrial dysfunction (Lassmann, van Horssen & Mahad 2012). These represent some major contemporary hypotheses on MS pathogenesis, but certainly more theories are investigated.

Therapeutic agents for multiple sclerosis reduce relapses, but do not achieve structural recovery

From a therapeutic perspective, MS relapse rates can be prevented to some extent, but no agent currently available definitely halts progression or promotes structural recovery of the inflicted injury. Relapse rates in relapsing-remitting MS are decreased on interferon β-1a, interferon β-1b or glatiramer acetate (PA et al. 2001, Loredana et al. 2012). Second-line treatments for patients who respond poorly or suffer from substantial side-effects include (addition of) natalizumab (Polman et al. 2006, Rudick et al. 2006) and fingolimod (Kappos et al. 2010, Cohen et al. 2010). Natalizumab is a selective antibody against the *α*4 integrin receptor on leukocytes and effectively prevents their entry into the brain (Yednock et al. 1992), but it can also reactivate the John Cunningham (JC) polyomavirus to cause progressive multifocal leukoencephalopathy (Clifford et al. 2010), and hence is prescribed with caution. Fingolimod modifies the sphingosine-1-phosphate receptor on lymphocytes to prevent them from leaving lymph nodes (Mandala et al. 2002) and is the first oral drug for MS patients, but clinical experience with fingolimod is still limited and requires careful monitoring of eventual risks that possibly become evident in clinical practice, such as the development of tumefactive MS or rapid disease progression on switching to fingolimod (Kinney et al. 2013). Expected first-line additions to this therapeutic arsenal comprise the oral immunomodulators laquinimod (Comi et al. 2012) and dimethyl fumarate (BG-12) (Fox et al. 2012, Gold et al. 2012). Further, the possible usefulness of alemtuzumab, a monoclonal antibody against the CD52 protein on mature lymphocytes, is investigated (Cohen et al. 2012, Coles et al. 2012), as well as the additional value of the antibody rituximab against CD20 on B-cells (Dian et al. 2011). Although not all of these drugs have been tested or subjected to long-term monitoring in progressive MS, their contribution to structural recovery of CNS damage is likely modest (Franklin et al. 2012a), allowing for disability to accumulate in MS patients. Substantial therapeutic benefit could therefore be expected from strategies that overcome demyelination and axonal degeneration. Given the importance of myelin for proper axonal function and axon protection (Franklin et al. 2012a, Bruce, Zhao & Franklin 2010) (see above), one of these strategies would be to restore myelin sheaths to

demyelinated axons (remyelination), thereby promoting functional recovery after demyelination (Duncan et al. 2009). In addition, treatments that promote remyelination would likely also offer a therapy for other demyelinating conditions, such as Pelizaeus-Merzbacher disease (see above).

EXPERIMENTAL MODELS TO STUDY REMYELINATION

Culture systems and animal models

Insights into why remyelination fails will be facilitated by a thorough understanding of the biology of myelin biogenesis and remyelination. Remyelination is a physiological process in the CNS, occurring spontaneously for example after demyelination in traumatic injury (Smith, Jeffery 2006). Remyelination is executed by local OPCs (Zawadzka et al. 2010) (see above), while SVZ OPCs contribute predominantly to remyelination of lesions in their proximity (Menn et al. 2006b). Our comprehension of the biology of complete remyelination grows from studying OPCs in several models. To start with, OPCs are examined *in vitro*, with the major culture systems making use of a) primary OPCs and oligodendrocytes, b) co-cultures of OPCs with dorsal root ganglion neurons, c) spinal cord cultures, to which an astrocyte feeding layer may be added and d) cerebellar slice cultures. In the latter system, demyelination can be induced by adding lysolecithin to the culture medium, which is subsequently followed by remyelination over two weeks. Despite their artificial nature, observations from these models are generally in agreement with *in vivo* findings, and, from a practical perspective, genetic manipulation and controlled modulation of other experimental conditions are relatively easy to accomplish (reviewed in: (Jarjour et al. 2012, van der Star et al. 2012)). Whereas the culture systems thrive on cells from neonatal mice and rats, culturing human adult OPCs and oligodendrocytes is also attempted (Roy et al. 1999). Secondly, remyelination can also be examined *in vivo,* for example in rodent toxin-induced animal models. These models induce demyelination by administering toxins harmful to oligodendrocytes, which evokes an endogenous response to achieve remyelination over time, usually several weeks. Examples are a) the dietary cuprizone model, resulting in demyelination in distinct brain regions, most notably the corpus callosum but also other white and gray matter areas (Matsushima, Morell 2001, Kipp et al. 2009), and b) the direct injection of lysolecithin or ethidium bromide into white matter (Woodruff, Franklin 1999, Blakemore, Franklin 2008). In other *in vivo* models, rodents with innate mutations in myelin genes ('myelin mutants') are studied, providing valuable insights into developmental myelination, which resembles remyelination in many ways (Franklin, Hinks 1999, Fancy et al. 2011b) (see below). A well-known example is the *shiverer* mouse that suffers from a mutation in the MBP gene and hence fails to generate compact myelin (reviewed in: (Duncan, Kondo & Zhang 2011)). Furthermore, additional models are tested for their eventual merits, with examples being zebrafish myelination (Buckley, Goldsmith & Franklin 2008, Czopka, Lyons 2011) and targeted cell ablation in Xenopus (Kaya et al. 2012). Our understanding of why remyelination fails in MS will improve by using these models in conjunction with studies on post-mortem human MS lesions and models of the MS disease, such as Theiler's murine encephalomyelitis virus (TMEV) model (van der Star et al. 2012,

Tsunoda, Fujinami 2010) and the EAE animal model for relapsing-remitting MS. The EAE model is characterized by adaptive immune-mediated demyelination in a chronic or relapsing pattern as a result of immunizing animals with myelin-derived antigens, for instance MOG, MBP or PLP, or constitutive expression of a myelin receptor on T-cells. Relapses are followed by remissions, in which remyelination is evident (Batoulis et al. 2011). Remyelination also occurs to a variable extent in a proportion of MS lesions (Patrikios et al. 2006), as is evident from thinner myelin sheaths relative to the axon diameters and shorter internode lengths (reviewed in: (Franklin, ffrench-Constant 2008)). However, remyelination is unsuccessful in chronic demyelinated lesions despite a relative excess of OPCs in most lesions (Wolswijk 1998, Wolswijk 2002). Why, then, does remyelination eventually fail in MS?

REMYELINATION IN MULTIPLE SCLEROSIS BY ENDOGENOUS CELLS

Remyelination commences with the activation of oligodendrocyte progenitor cells

Spontaneous remyelination requires OPCs to a) become activated, b) proliferate and migrate towards the lesion ('recruitment') and c) differentiate into myelinating oligodendrocytes (reviewed in: (Franklin, ffrench-Constant 2008)). Activation of OPCs may be achieved by soluble signals from activated astrocytes and microglia (Rhodes, Raivich & Fawcett 2006) and is characterized by alterations in the morphology of OPCs (Reynolds et al. 2002). Further, activated OPCs re-express factors important for myelination, for example Shh (Ferent et al. 2013) and transcription factors such as Nkx2.2 and Olig2 (Watanabe, Hadzic & Nishiyama 2004, Talbott et al. 2005) as well as Olig1, which is essential for remyelination (Arnett et al. 2004). Indeed, coordination of subsequent proliferation and migration involves recapitulation of many intracellular and extracellular factors that guide developmental myelination (Franklin, Hinks 1999, Fancy et al. 2011a) (see above). For example, the familiar mitogens FGF-2 and PDGF-AA (Williams et al. 2007), and ECM molecules such as laminin (van Horssen et al. 2006, Satoh, Tabunoki & Yamamura 2009) are upregulated in demyelinated lesions.

Several prerequisites for successful oligodendrocyte progenitor cell maturation

In order for activated OPCs to proceed through subsequent stages of remyelination successfully (Fig. 2), several prerequisites have to be met. These include the presence of astrocytes, possibly favourably activated (Talbott et al. 2005, Blakemore, Gilson & Crang 2003), microglia (Li et al. 2005) and macrophages (Kotter et al. 2005). Which elements in the presence of these cells are necessary for remyelination and which are redundant is not yet clear. Many beneficial elements may be similar to those required for regeneration of other tissues, such as salamander limbs or cutaneous wounds that also need innate immune activity (Deonarine et al. 2007, Godwin, Pinto & Rosenthal 2013). Some specific helpful effects of innate immune activity for remyelination have been identified. An example is tumor necrosis factor-α (TNF-α) (Arnett et al. 2001), which is synthesized, among others, by microglia/ macrophages on iron efflux of astrocytes (Schulz, Kroner & David 2012). To enhance remyelination, TNF-α signals to the TNF receptor 2 (TNFR-2) on astrocytes, which results in increased

expression of CXCL12, a chemokine that promotes remyelination (Patel et al. 2012) by stimulation of its receptor CXCR7 on OPCs (Gottle et al. 2010). In line with these beneficial effects, inhibition of TNF-α enhances the number of relapses in MS (The Lenercept Multiple Sclerosis Study Group, 1999). However, TNF-α may also signal to the TNF receptor 1 (TNFR-1) on OPCs and other glial cells, which is associated with cell death (Tartaglia et al. 1991, Tchelingerian et al. 1995). In accordance, a blocking antibody against soluble TNF-α, which predominantly signals to TNFR-1, is associated with recovery and remyelination in EAE (Brambilla et al. 2011). Other (predominantly) favorable immune molecules include IL-1β (Mason et al. 2001), activin-A from M2 polarized microglia (Miron et al. 2013) and endothelin-2, which signals to the endothelin receptor B on OPCs (Yuen et al. 2013). Innate immune activity is further considered important for the removal of myelin debris. Whether myelin debris is a major obstacle for remyelination in MS remains to be established, but myelin debris has the capacity to inhibit remyelination (Kotter et al. 2006). Phagocytosis of myelin debris benefits from a) recruitment of microglia by astrocytes (Skripuletz et al. 2013) and b) competent monocytes, which in this study meant 'juvenile' rather than 'aged' monocytes (Ruckh et al. 2012). Indeed, remyelination likely becomes less efficient with age due to a change in the innate immune response to demyelination (Ruckh et al. 2012, Zhao, Li & Franklin 2006). Possibly reflecting the availability of some of the factors just discussed, the capacity to remyelinate also differs between brain regions. For example, white matter lesions remyelinate less extensively than grey matter lesions (Albert et al. 2007). This may be related to the observation that white matter lesions display more pronounced astrogliosis, which refers to an excess of hypertrophic, scarring astrocytes thought to frustrate regeneration (Chang et al. 2012).

Remyelination failure: insufficient mature oligodendrocytes

In MS lesions, OPCs may not meet with sufficient prerequisites for remyelination. In addition, MS lesions contain factors that specifically impair remyelination. Contemporary thoughts focus on two major obstacles that OPCs meet during their development towards remyelinating oligodendrocytes in MS. First, in a subset of MS lesions, insufficient migration and proliferation of OPCs likely accounts for remyelination failure. Defective recruitment could be an effect of repeated assaults on OPCs as such during the MS disease process (Niehaus et al. 2000, Mason et al. 2004). However, a more substantial barrier likely results from the inappropriate expression of factors repellent to OPCs, such as certain semaphorins (Williams et al. 2007, Boyd, Zhang & Williams 2013). An upregulation of chemorepellent semaphorins corresponds to remyelination failure in up to a third of MS lesions (Williams et al. 2007, Boyd, Zhang & Williams 2013), possibly predominantly larger ones (reviewed in: (Franklin, ffrench-Constant 2008)). In general, though, OPCs are considered capable of replenishing their numbers on repetitive demyelination (Penderis, Shields & Franklin 2003), and are abundantly present in the majority of both active and chronic MS lesions (Wolswijk 1998, Wolswijk 2002, Schonrock et al. 1998, Kuhlmann et al. 2008). Therefore, the second hypothesis attributes remyelination failure largely to defective differentiation of OPCs and further maturation

(Franklin, ffrench-Constant 2008, Kuhlmann et al. 2008, Franklin 2002). In support of this, several factors that inhibit OPC differentiation have been identified and, in some cases, detected in MS lesions. For example, several ECM proteins are not transiently, but permanently upregulated in MS. These include chondroitin sulfate proteoglycans (CSPGs) (Sobel, Ahmed 2001) and high molecular weight hyaluronan (Back et al. 2005), both inhibitory for remyelination (Back et al. 2005, Lau et al. 2012). For high molecular weight hyaluronan, the mechanism involves triggering activity from the hyaluronidase PH20 in OPCs, resulting in expression of adverse hyaluronan digestion products (Preston et al. 2013). Several other ECM proteins are also prominently expressed in demyelinated lesions, including fibronectin (Satoh, Tabunoki & Yamamura 2009, Sobel, Mitchell 1989, Zhao et al. 2009, Hibbits et al. 2012, Stoffels et al. 2013) (this thesis). However, among these are also ECM proteins with a known stimulatory influence on myelination, such as laminin (Colognato et al. 2002, van Horssen et al. 2006), whereas tenascin-C and tenascin-R are depleted from acute, but not chronic lesions (Gutowski, Newcombe & Cuzner 1999). Therefore, the overall effects of ECM and other environmental signals, as well as their mutual interactions remain to be established, although the net outcome is ultimately unfavourable, given the failure of remyelination. Additional inhibitory signals from the environment include PSA-NCAM from axons (Charles et al. 2000), and Jagged from astrocytes, which signals to Notch1 on OPCs and keeps them in an undifferentiated state by sustaining expression of the transcription factor Hes5 (John et al. 2002). Indeed, other transcription factors from the derepression model, which were discussed above, are also persistently upregulated in MS OPCs, such as Tcf4, resulting from aberrant canonical Wnt activity via Axin2 (Fancy et al. 2009, Fancy et al. 2011c). OPCs further remain undifferentiated due to sustained Lingo-1 expression (Mi et al. 2007, Mi et al. 2009)) (reviewed in: (Huang et al. 2011a)). Finally, adverse innate immune activity likely contributes to remyelination failure, with unfavourable macrophage/ microglia polarization (Miron et al. 2013) and, possibly, mainly detrimental macrophage/ microglia activity (reviewed in: (Rawji, Yong 2013)).

The dysregulation hypothesis of remyelination failure

Interestingly, many of the factors that may impede remyelination in MS are involved in the timing of developmental myelination (discussed above) (Franklin, Hinks 1999, Fancy et al. 2011a). Therefore, their sustained activity in MS suggests a dysregulation mechanism of remyelination failure. This dysregulation mechanism describes that, in contrast to molecular events in developmental myelination, the timing and sequence of events guiding OPCs towards remyelination is inappropriate, thereby disturbing successful remyelination (Franklin, ffrench-Constant 2008, Fancy et al. 2011a, Franklin 2002). Of interest, impairment of glial cell maturation is also apparent in leukoencephalopathy with vanishing white matter. This genetic disease is clinically characterized by spasticity and progressive cerebellar ataxia, often with onset at childhood. Pathologically, myelin degeneration is evident, but with abundance of oligodendrocytes, and relatively few and immature, dysmorphic astrocytes (van der Knaap, Pronk & Scheper 2006). Interestingly, high molecular weight

hyaluronan accumulates here, as well as in MS lesions, and thus may frustrate maturation of oligodendrocytes and astrocytes, thereby hampering regeneration (Bugiani et al. 2013).

In addition to the continuous expression of obstructing factors, remyelination is also dysregulated in MS from a relative deficiency of various stimulants for remyelination. For example, stimulation of estrogen receptors promotes remyelination by activation of the PI3k/Akt/mTOR and ERK1/2 pathways in OPCs (Xiao et al. 2012, Hirahara et al. 2013, Kumar et al. 2013), and plasma levels of estrogen tend to be lower when MS activity is more severe (Dwosh et al. 2003). Also, progesterone and prolactin, both important hormones during pregnancy or lactation, promote remyelination (Ibanez et al. 2004, Gregg et al. 2007), and provide a possible mechanism for why pregnancy is associated with MS remission (Confavreux et al. 1998). Furthermore, testosterone promotes remyelination via interactions with both the brain immune system and the neural androgen receptor on neurons and glia (Hussain et al. 2013), whereas a relative testosterone deficiency is associated with progressive MS in males (Safarinejad 2008) as well as a higher MS lesion load in females (Tomassini et al. 2005). Certainly, these steroid hormones are versatile molecules that also exert distinct effects on the immune system, but given their ability to promote remyelination, further investigations are warranted. An additional hormone that may benefit remyelination is the thyroid hormone (Fernandez et al. 2004). Thyroid hormone signals via its nuclear receptor that functions by engaging in heterodimeric association with other nuclear receptors. Of interest, another nuclear receptor, retinoid X receptor gamma (RXRγ), appears to be a positive regulator of OPC differentiation that is expressed in MS lesions by OPCs, macrophages/microglia and astrocytes. This suggests that remyelination could also be supported by administration of appropriate ligands for RXRγ, such as rexinoids, or for the heterodimeric permissive partners of RXRγ, which are likely the liver X receptor (LXRs) and peroxisome proliferator activator proteins (PPAR) (Huang et al. 2011a, Huang et al. 2011b). Lastly, although local levels of several important growth factors are not precisely known for MS lesions, some may become deficient in chronic lesions. These may include neuregulin (Viehover et al. 2001) as well as PDGF, which enhances remyelination of chronic experimental white matter lesions (Vana et al. 2007) and, possibly, epidermal growth factor (EGF) (Aguirre et al. 2007).

In summary, in order to enhance endogenous remyelination, the majority of MS lesions will likely benefit from promoting differentiation of OPCs, possibly even into Schwann cells (Zawadzka et al. 2010, Fancy et al. 2011a, Crawford, Chambers & Franklin 2013). A thorough understanding of which negative factors are overexpressed or dominant, and which positive factors are deficient or subdominant in MS lesions will contribute to identifying molecular targets to promote endogenous remyelination. One such a negative factor, Lingo-1, is currently targeted in an early-phase clinical trial with a humanized anti-Lingo-1 monoclonal antibody to possibly benefit endogenous remyelination (Mi, Pepinsky & Cadavid 2013).

The other approach: remyelination by transplantation of exogenous cells

Finally, this overview on the background and current status of strategies to promote remyelination would be rather incomplete without briefly addressing the possibility of stem cell transplantation. This approach aims at delivering exogenous stem cells to demyelinated areas so that they may promote or execute local remyelination. Considering the rationale behind this design, it seems more logical to use small-molecule therapies in MS for boosting endogenous OPCs, equally well-equipped to remyelinate (Franklin, ffrench-Constant 2008). However, mitotic exhaustion of OPCs could be an obstacle for remyelination of chronic lesions, although this is not well-established (Robins et al. 2013), and then the supply of fresh, mitotic competent cells could add benefits, albeit a potential risk of tumorigenesis as well (Goldman, Nedergaard & Windrem 2012). Also, transplanted cells may well add further beneficial support via 'bystander effects' and immune modulation (reviewed in: (Martino et al. 2010, Rivera, Aigner 2012)).

Major outstanding issues for exogenous strategies are to resolve a) where cells should be transplanted to, and b) which cells they should be. As for the location, because intra-lesion injection is anatomically and practically complex, more benefit is expected from intraventricular or intravenous delivery (Franklin, ffrench-Constant 2008). The major candidate cells to be transplanted include allogenic cells, such as human embryonic stem cells (Nistor et al. 2005), fetal neural stem cells or glial progenitor cells (Windrem et al. 2008). Alternatively, autologous cells may be used, including haematopoietic (Mancardi 2009) and mesenchymal stem cells (reviewed in: (Rivera, Aigner 2012)), olfactory ensheathing cells (Franklin 2003) as well as induced pluripotent stem cells (iPSCs) (Takahashi, Yamanaka 2006). For allogenic human neural stem cells, relative safety and enhanced myelination is suggested from two pioneering studies in infants with Pelizaeus-Merzbacher disease and its *shiverer* mouse animal model (Gupta et al. 2012, Uchida et al. 2012). However, in general, the use of autologous cells would likely reduce the risk of graft rejection and need for immune suppression (Goldman, Nedergaard & Windrem 2012). Haematopoietic stem cells reduce inflammatory activity in MS (Mancardi, Saccardi 2008), but do not contribute to structural recovery (Metz et al. 2007). Mesenchymal stem cells are currently tested in clinical trials (Connick et al. 2012), whereas olfactory ensheathing cells benefit local pathology on transplantation after spinal cord injury in dogs (Granger et al. 2012). Finally, iPSCs from rodent fibroblasts can be differentiated into oligdenderocyte lineage cells to create functional oligodendrocytes both *in vitro* and *in vivo* (Czepiel et al. 2011, Najm et al. 2013, Yang et al. 2013), offering an encouraging prospective on this possibility for human fibroblasts. In summary, exogenous cell therapies may offer therapeutic benefit in distinct ways, but carry risks as well and have not proven superiority over endogenous strategies. This warrants intense investigation in both directions to hopefully accelerate the availability of (complementary) therapies, ensuring structural recovery and reducing progression in MS.

SCOPE OF THIS THESIS

In order to identify molecular targets for promoting endogenous remyelination, an understanding of how remyelinating OPCs are affected by extrinsic factors from the MS environment is pivotal (chapter 1). In this thesis, the ECM molecule fibronectin was studied with the aim of identifying how fibronectin affects CNS remyelination and its failure in MS. Hereto, fibronectin expression was characterized after demyelination in experimental animal models and in MS, revealing a correlation between fibronectin aggregation and failure of remyelination. Therefore, functional consequences of fibronectin aggregation for remyelination were evaluated (chapter 2). Because fibronectin is expressed on demyelination seemingly 'by default', an initially beneficial role in complete CNS remyelination was presumed. Hence, conditional knockout of plasma fibronectin and cellular fibronectin from astrocytes was induced to study how this affects complete, physiological remyelination (chapter 3). In addition to OPCs, other cells in MS lesions likely also receive signals from fibronectin aggregates. In chapter 4, therefore, the effects of fibronectin aggregates on microglia and macrophages were assessed. As indicated, fibronectin expression on myelin injury (demyelination) seemed a common response, and in fact, tissue injury in general evokes synthesis of a fibronectin matrix. A literature review on fibronectin expression in tissue injury was therefore undertaken in chapter 5. From this review, it became evident that improper degradation of fibronectin harms tissue regeneration also in chronic wounds and osteoarthritis, suggesting that degradation of fibronectin is a prerequisite for complete regeneration of tissue (chapter 5). Finally, main conclusions and future perspectives are summarized (chapter 6).

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Chapter 2

Fibronectin aggregation in multiple sclerosis lesions impairs remyelination

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ABSTRACT

Remyelination following central nervous system demyelination is essential to prevent axon degeneration. However, remyelination ultimately fails in demyelinating diseases such as multiple sclerosis. This failure of remyelination is likely mediated by many factors, including changes in the extracellular signalling environment. Here, we examined the expression of the extracellular matrix molecule fibronectin on demyelinating injury and how this affects remyelination by oligodendrocytes progenitors. In toxin-induced lesions undergoing efficient remyelination, fibronectin expression was transiently increased within demyelinated areas and declined as remyelination proceeded. Fibronectin levels increased both by leakage from the blood circulation and by production from central nervous system resident cells. In chronically demyelinated multiple sclerosis lesions, fibronectin expression persisted in the form of aggregates, which may render fibronectin resistant to degradation. Aggregation of fibronectin was similarly observed at the relapse phase of chronic experimental autoimmune encephalitis, but not on toxin-induced demyelination, suggesting that fibronectin aggregation is mediated by inflammation-induced demyelination. Indeed, the inflammatory mediator lipopolysaccharide induced fibronectin aggregation by astrocytes. Most intriguingly, injection of astrocyte-derived fibronectin aggregates in toxin-induced demyelinated lesions inhibited oligodendrocyte differentiation and remyelination, and fibronectin aggregates are barely expressed in remyelinated multiple sclerosis lesions. Therefore, these findings suggest that fibronectin aggregates within multiple sclerosis lesions contribute to remyelination failure. Hence, the inhibitory signals induced by fibronectin aggregates or factors that affect fibronectin aggregation could be potential therapeutic targets for promoting remyelination.

INTRODUCTION

Inflammation-mediated loss of myelin (demyelination) and incomplete remyelination are pathological hallmarks of multiple sclerosis. Remyelination is essential for both restoration of saltatory conduction and axonal protection (Franklin, ffrench-Constant 2008). Although remyelination occurs in early stages of multiple sclerosis, it declines as the disease progresses, resulting in chronically demyelinated plaques and axonal loss (Goldschmidt et al. 2009). Oligodendrocyte progenitors, the cells responsible for CNS remyelination (Zawadzka et al. 2010), are present in most multiple sclerosis lesions, but ultimately fail to differentiate into mature myelinating oligodendrocytes, which results in remyelination failure (Franklin, ffrench-Constant 2008, Kuhlmann et al. 2008).

Migration and proliferation of oligodendrocyte progenitor cells and their differentiation into myelinating oligodendrocytes are regulated by many factors, including the extracellular matrix (Baron, Colognato & ffrench-Constant 2005). For example, laminin-2 provides oligodendrocytes with signals for both survival (Colognato et al. 2002, Baron et al. 2003) and myelination (Buttery, ffrench-Constant 1999, Relvas et al. 2001, Siskova et al. 2006). In contrast, fibronectin promotes proliferation, but reduces myelin-like membrane formation (Baron, Colognato & ffrench-Constant 2005, Buttery, ffrench-Constant 1999, Siskova et al. 2006, Maier et al. 2005, Siskova et al. 2009). Following CNS injury, the extracellular matrix is extensively remodelled, which is reflected in altered expression profiles of extracellular matrix molecules (Sobel, Mitchell 1989, Gutowski, Newcombe & Cuzner 1999, Back et al. 2005, van Horssen et al. 2005, van Horssen et al. 2006, Satoh, Tabunoki & Yamamura 2009). Therefore, aberrant extracellular matrix signals in the injury environment may inhibit oligodendrocyte maturation, contributing to remyelination failure in multiple sclerosis lesions. Indeed, while absent from healthy adult human tissue, expression of fibronectin is upregulated in multiple sclerosis lesions, particularly around blood vessels (Sobel, Mitchell 1989, van Horssen et al. 2005), and also in the CNS parenchyma (van Horssen et al. 2006). Furthermore, astrocyte-derived high molecular weight hyaluronan, which inhibits oligodendrocyte maturation, appears in chronic demyelinating multiple sclerosis lesions (Back et al. 2005). Laminin expression is also increased in multiple sclerosis lesions (van Horssen et al. 2005). Therefore, the overall effects of lesion-induced changes in the extracellular matrix on oligodendrocyte progenitor cells remain to be established.

Here, we further characterized the nature of fibronectin signaling to oligodendrocyte progenitor cells in multiple sclerosis lesions, and how this fibronectin signalling might affect remyelination. We show that fibronectin specifically localizes in areas of demyelination, and identify cellular sources for its expression. In experimental demyelination, fibronectin is cleared on remyelination, whereas fibronectin aggregates and therefore persists in chronic multiple sclerosis lesions and chronic relapsing experimental autoimmune encephalomyelitis (EAE). Importantly, intralesion injection of fibronectin aggregates inhibits oligodendrocyte differentiation and remyelination in toxin-induced demyelinated lesions, which implies that aggregation of fibronectin contributes to remyelination failure in multiple sclerosis lesions.

MATERIALS AND METHODS

Multiple sclerosis lesions

Tissues were obtained from the Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. Studies were performed on brain material taken at autopsy from nine healthy subjects (without clinical or histological signs of neurological disease), eight subjects with (chronic) active multiple sclerosis lesions, nine with chronic inactive multiple sclerosis lesions and two with multiple sclerosis shadow plaques (Maier, Baron & Hoekstra 2007). Samples were selected according to their activity status as determined by MRI, split in half and either immediately frozen in liquid nitrogen or fixed in formaldehyde and paraffin embedded. Control white matter did not show any histological signs of inflammation and demyelination. Demyelinated lesions were identified by Luxol fast blue histochemistry and proteolipid protein staining. Active lesions were characterized by their indistinct margin, hypercellularity, intense perivascular T lymphocyte infiltration (CD3) and presence of hypertrophic astrocytes (glial fibrillary acidic protein, GFAP) and macrophages (CD68) in the centre of the lesions. Chronic active lesions were classified on the basis of a hypocellular lesion centre with fibrous astrocytes and some macrophages, sharp lesion border and a broad rim of macrophages. Chronic inactive lesions contained a hypocellular centre without macrophages and lymphocytes, and a sharp lesion border. Shadow plaques were characterized by a slightly reduced proteolipid protein expression compared with the surrounding normal-appearing white matter, without abundant expression of CD68. Notably, remyelinated areas, as determined by Luxol fast blue or proteolipid protein staining, were not visible in the other multiple sclerosis lesions analysed. For immunohistochemical analysis, sections were deparaffinized and subjected to antigen retrieval followed by fluorescent detection as described previously (Stancic et al. 2011). Control sections from which the primary antibody was omitted showed low non-specific binding. Sections were analysed using a conventional fluorescence microscope (Olympus AX70) equipped with analySIS software. For western blot and reverse transcription–PCR analysis, samples were homogenized and extracted for protein and total RNA as described (Maier, Baron & Hoekstra 2007). All material was collected from donors whose written informed consent for brain autopsy and the use of the material and clinical information for research purposes has been obtained by the Netherlands Brain Bank.

Toxin-induced demyelination

Lesions were induced in spinal cord white matter or in the caudal cerebral peduncle of 8–10-week-old female Sprague Dawley rats (Harlan) by injection of 1 ml of 1% lysolecthin (Sigma Aldrich) or 4 ml of 0.01% ethidium bromide (VWR), respectively (Fancy, Zhao & Franklin 2004, Zhao, Li & Franklin 2006). At the desired time points, animals were sacrificed and tissue processed as described previously (Fancy, Zhao & Franklin 2004, Zhao, Li & Franklin 2006, Woodruff, Franklin 1999). The control spinal cord tissues consisted of similar fragments from unlesioned thoracic segments of spinal cord. The intralesion injection of fibronectin aggregates was performed at 7 days post lesion into lysolecithin-induced lesions in rat dorsal funiculus of spinal cord. A volume of 2 ml of fibronectin aggregates (0.3

mg/ml) was injected using a Hamilton syringe with a pulled glass tip attached. These animals were sacrificed 7 days after aggregate injection, i.e. 14 days post lesion. Experiments were performed in compliance with UK Home Office regulations.

Chronic relapsing experimental autoimmune encephalomyelitis

Chronic relapsing EAE was induced with recombinant rat myelin oligodendrocyte glycoprotein in adult male Dark Agouti rats (Harlan, weight 230–250 g) as described previously (Ledeboer et al. 2003). Briefly, the rats were anaesthetized with isoflurane and immunized intradermally in the dorsal tail base with 75 mg of recombinant rat myelin oligodendrocyte glycoprotein (rrMOG1–125) emulsified in incomplete Freund's adjuvant (Difco) together with 10mM NaAc (pH 3.0). Control rats received incomplete Freund's adjuvant and NaAc only. Rats were weighed and examined daily for neurological symptoms of EAE that were scored on the following scale: 0, no clinical disease; 0.5, partial loss of tail tone; 1, complete tail atony; 2, paresis, partial hind limb paralysis; 3, complete paralysis of the hind limbs and/or lower part of the body; 4, moribund or dead due to EAE. Chronic relapsing EAE and incomplete Freund's adjuvant control animals were sacrificed when chronic relapsing EAE rats reached the peak clinical symptoms during the relapse (~25 days post injection) and spinal cords were processed as previously described (Maier et al. 2005, Ledeboer et al. 2003). All experimental procedures were approved of by the Animal Ethical Committee of the VU University.

Cell cultures

Primary glial cells: Primary glial cultures were generated from 1 to 3-day-old Wistar rats (Harlan) as described previously (Bsibsi et al. 2012). Isolated microglia and astrocytes were cultured in Dulbecco's modified Eagle medium/10% foetal calf serum in 10 cm dishes (1.0 x 10^6/dish) or 8-well Permanox chamber slides (15.000 cells/well; Nunc). Cells were left untreated or activated with 200 ng/ml ultrapure lipopolysaccharide (InvivoGen) for 48 h. Deposited astroglial matrices were prepared by water-lysis of astrocytes for 2 h at 37ºC. Isolated oligodendrocyte progenitor cells were cultured in Sato medium (Maier et al. 2005) in poly-L-lysine-coated 10 cm dishes (1.0 x 10^6/dish) or 8-well chamber slides (15.000 cells/well) with wells coated with astroglial matrices (see later in the text). The oligodendrocyte progenitor cells were first synchronized in Sato medium supplemented with platelet-derived growth factor AA (Peprotech; 10 ng/ml) and fibroblast growth factor-2 (Peprotech, 10 ng/ml) for 2 days. Differentiation was induced by growth factor withdrawal and cells were grown for 7 days in Sato medium containing 0.5% foetal calf serum.

Human astrocytes: Adult human astrocytes were isolated from post-mortem subcortical white matter of well-documented healthy subjects and patients with multiple sclerosis, as described previously (Bsibsi et al. 2012). Purity of the cultures was routinely verified by staining for GFAP (astrocytes). Astrocyte cultures used were at least 97% pure. The cells were plated in 10 cm dishes (1.0 x 10^6/dish) or 8-well chamber slides (10.000/well). Deposited astroglial matrices were prepared by water-lysis of astrocytes for 2 h at 37ºC.

Immunohistochemical analysis

Toxin-induced lesions: Frozen sections (12 mm) of spinal cord and caudal cerebral peduncle were permeabilized and blocked with phosphate-buffered saline (PBS) containing 5% normal donkey serum and 0.3% Triton™ X-100. Sections were incubated with the appropriate primary antibodies (Table 1) diluted in PBS containing 3% normal donkey serum overnight at 4°C. For double labelling immunohistochemistry, primary antibodies were incubated sequentially. After washing in PBS, the sections were incubated with appropriate Alexa Fluor© (488 or 594)-conjugated secondary antibodies (Invitrogen 1:500) at room temperature for 2 h, followed by 4',6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Following immunostaining, some slides were incubated in 0.1% Sudan Black solution (made in 70% ethanol) for 5 min at room temperature to stain myelin. Pale areas in the white matter indicate loss of myelin. Sudan Black staining has no detrimental effect on immunofluorescence. Sections were analysed with a Zeiss Axio Observer A1 fluorescent microscope. Chronic relapsing experimental autoimmune encephalomyelitis: Fresh frozen rat spinal cord sections (12 mm) were fixed with acetone for 20 min, washed in Tris-buffered saline, blocked in 5% milk in Tris-buffered saline with 0.5% TritonTM X-100 and 0.03% H2O2 and incubated with primary antibodies (Table 1) overnight at 4°C, followed by appropriate Alexa Fluor© (488 or 594)-conjugated secondary antibodies (1:400) for immunofluorescence. Sections were examined with a Leica confocal laser scanning microscope. For peroxidase-based analysis, sections were washed in

Table 1: Primary antibodies used during IHC, ICC and WB

	manufacturer	dilution WB	dilution IHC/ICC
anti-actin (mAb)	Sigma	1:1000	n.a.
anti-APC (CC1, mAb)	Calbiochem	n.a.	1:200
anti-CD3 (mAb)	Zymed	n.a.	1:100
anti-CD11b (MAC1, mAb)	Serotec	n.a.	1:100
anti-CD68 (mAb)	DAKO	n.a.	1:100
anti-CNP (mAb)	Sigma	1:500	n.a.
anti-EIIIA-fibronectin (IST9)	Abcam	n.a.	1:200
anti-fibronectin (pAb)	Millipore	1:1000	1:50-1:100
anti-GFAP (mAb)	Millipore	1:1000	1:500
anti-GFAP (pAb)	DAKO	n.a.	1:500
anti-laminin 1+2 (pAb)	Abcam	1:500	n.a.
anti-MBP (mAb)	Chemicon	1:100	1:25
anti-Nkx2.2 (mAb)	Hybridoma Bank Iowa	n.a.	1:100
anti-Olig2 (pAb)	Millipore	n.a.	1:1000
anti-OX6 (mAb)	Serotec	n.a.	1:1000
anti-PLP (mAb)	Serotec	n.a.	1:3000
TuJ1 (mAb)	kind gift of A. Frankfurter[1]	1:2000	n.a.
anti-vWF (mAb)	Serotec	n.a.	1:50
anti-vWF (pAb)	DAKO	n.a.	1:200

n.a., not applicable; mAb, monoclonal antibody; pAb, polyclonal antibody; 1) (Lee et al. 1990)

Tris-buffered saline and incubated for 2 h at room temperature with secondary biotinylated IgGs (Jackson ImmunoResearch, 1:400), followed by washes in Tris-buffered saline and incubation for 1 h at room temperature with avidin–biotin–peroxidase complex (Vector Laboratories, 1:400). After washes in Tris-buffered saline and 50mM Tris–HCl (pH 7.6), immunoreactivity was visualized using 0.5 mg/ml of diaminobenzidene (Sigma) in 50mM Tris–HCl (pH 7.6).

Immunocytochemical analysis

Oligodendrocytes were fixed with 4% paraformaldehyde for 20 min and permeabilized with ice-cold methanol for 10 min. After a 30-min block with 4% bovine serum albumin, cells were incubated for 60 min with primary antibodies (Table 1) in 4% bovine serum albumin. Cells were washed three times with ice-cold PBS and incubated for 25 min with appropriate tetramethyl rhodamine isothiocyanateconjugated antibodies (Jackson ImmunoResearch). Nuclei were stained with DAPI (1 mg/ml) and 1,4-diazabicyclo[2.2.2]octane-containing mounting medium was added to prevent image fading. For staining of astroglial matrices, fixation and permeabilization steps were omitted. Oligodendrocytes were characterized by their morphology, and in each experiment at least 500 cells were scored as myelin basic protein (MBP)-positive or -negative. In addition, positive cells bearing MBP-positive membranous structures spread between the cellular processes were identified as membrane sheet forming, irrespective of the extent of sheet formation.

Reverse transcription–polymerase chain reaction

Total RNA was isolated from cells and tissue homogenates using the RNeasy_ Mini kit (Qiagen). Total RNA from tissue (0.5 mg) or cells (1.0 mg) was reverse transcribed in the presence of oligo(dT)12–18 and dNTPs (Invitrogen) with SuperScript_ II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting complementary DNA was amplified using primers specific to the different proteins (Table 2). Cycling conditions were optimized and PCR products were resolved by agarose gel electrophoresis. Changes in gene expression were analysed by Scion Image Software.

Table 2. Primer sequences used during RT-PCR

	sense	anti-sense	length (bp)
Actin Hs/Rn	ACCACACAGCTGAGAGGGAAATC	GGTCTTTACGGATGTCAACG	276
CD68 Hs	TATTGCTTTCTGCATCATCC	TTTAGTAGAGACAGGGTTTCAC	303
Cyclophilin Rn	CTCGTGACCCCTCTTTCC	CAT TATTTTCCTCATTTCCCCT	522
Fn1 Hs	GAGGAGAGTGGAAGTGTGAG	TTGTGGTTGTTGTATAGGAAGG	496
Fn1 Rn	AGCCC TTACAGTTCCAAGTTCC	CCATT CAATTCATTGCATCG	1077
GFAP Hs	CTCCAACTAACAAGAAACTCAC	GGCTCCAATCTATAATCCCA	412

Hs, homo sapiens, Rn, rattus norvegicus

***In situ* hybridization**

To generate a complementary RNA probe for fibronectin, complementary DNA acquired using fibronectin primers (Table 2) was cloned by reverse transcription–PCR and inserted into the pCRII-TOPO plasmid, using the TOPO© TA Cloning© Kit (Invitrogen) according to the manufacturer's instruction. The probe is expected to recognize all variants of fibronectin messenger RNA. The digoxigenin-labelled proteolipid protein probe was generated as described (Chari et al. 2006). The details for probe labelling and staining procedures have been described in previous studies (Sim et al. 2002, Zhao et al. 2008).

Deoxycholate (in)solubility assays and preparation of fibronectin aggregates

Tissue homogenates (multiple sclerosis and chronic relapsing EAE) were incubated with deoxycholate buffer [2% deoxycholate and Complete Mini protease inhibitor cocktail (Roche) in 20mM Tris–HCl, pH 8.3], for 30 min on ice. Proteins from lysolecithin-induced lesions were extracted from 30-mm thick slices through oscillation for at least 3 h in deoxycholate buffer. Deposited astroglial matrices were prepared by water-lysis of astrocytes for 2 h at 37ºC. The efficiency of lysis was verified by DAPI staining, and only matrices without visible nuclei were used. Deposits were scraped in ice-cold deoxycholate buffer and further solubilized for 30 min on ice. Protein concentrations were determined by a Bio-Rad DC protein assay (Bio-Rad Laboratories) using bovine serum albumin as standard. For biochemical analysis, deoxycholate-soluble and -insoluble fractions from equal protein amounts of the deoxycholate extracts were separated by centrifugation at 13.000 rpm for 20 min at 4ºC. The deoxycholate-insoluble pellets were dissolved in 2% SDS in 20mM Tris–HCl, pH 8.8, whereas the deoxycholate-soluble supernatant was concentrated by trichloroacetic acid precipitation. For intralesion injections, the aggregates present in the deoxycholate-insoluble fraction were dialyzed against PBS for 24 h at 4ºC. After dialysis, the protein content was determined and the presence of aggregates confirmed by western blot.

Lactate dehydrogenase and MTT assay

Oligodendrocyte progenitor cells were plated in 24-well plates (Nunc) at a density of 50.000 cells in 500 ml culture medium. Oligodendrocytes were treated with fibronectin aggregates (5.0 mg/ml of the deoxycholate-insoluble fraction of rat astrocyte-derived deposits dialyzed against PBS) at the onset of differentiation (oligodendrocyte progenitor cells), 3 days (immature oligodendrocytes) or 7 days (mature oligodendrocytes) after initiating differentiation. After 3 days, the medium (lactate dehydrogenase assay) and cells [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT] were analysed. To determine the cytotoxicity of deoxycholateinsoluble fibronectin aggregates, the release of lactate dehydrogenase into the medium was measured using a commercial lactate dehydrogenase assay kit (Roche) according to manufacturer's instructions. The effect on cell viability was determined with an MTT assay. Briefly, after medium was removed for the lactate dehydrogenase assay, cells were incubated with MTT diluted in culture medium (0.5 mg/ml,

Sigma) for 3–4 h. MTT-formazan crystals were collected in dimethyl sulphoxide and absorption was measured at 560 nm. Cytotoxicity (lactate dehydrogenase) and cell viability (MTT) are expressed as the percentage of vehicle-treated (PBS) cells, which was set at 100%.

Electron microscopy analysis

The animals were perfused with 4% glutaraldehyde and lesion containing spinal cord was coronally sliced at ~1mm thickness and fixed with osmium tetroxide overnight before being subjected to a standard protocol of epoxy resin embedding (Zhao et al. 2008). Ultrathin sections of the lesion site were produced at transverse orientation and examined with a Hitachi H-600 electron microscope. Myelination of axons in the lesion was analysed for g-ratio, which is calculated as the diameter of axons divided by the diameter of axons with surrounding myelin sheaths.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blotting

Equal amounts of proteins (tissue homogenates, cultured cells and reducing conditions) or volume [deoxycholate-(in)solubility assays, non-reducing conditions] were loaded onto 6 or 8% SDS-PAGE gels and subjected to western blot analysis as described previously (Bsibsi et al. 2012). The signals were detected using the Odyssey© Infrared Imaging System (Li-Cor Biosciences) and analysed using Odyssey V3.0 analysis software.

RESULTS

Cellular fibronectin expression is transiently increased in toxin-induced demyelination

Fibronectin effects on regeneration have been studied in various tissues (Scanzello, Plaas & Crow 2008). To assess whether on demyelination, changes in the extracellular matrix environment include fibronectin, we first examined the profile of fibronectin expression following toxin-induced demyelination. Injection of lysolecithin into spinal cord white matter creates focally demyelinated lesions without significant axonal loss (Blakemore, Franklin 2008). These lesions undergo spontaneous remyelination, involving the activation of oligodendrocyte progenitor cells and their recruitment into the demyelinated area (0–10 days post lesion), and their subsequent differentiation and myelin sheath formation (10–21 days post lesion) (Zhao, Li & Franklin 2006). In this model, the lesion area is characterized by an increased cellularity, which reflects well the area of demyelination as visualized with a Sudan Black myelin stain (Fig. 1A). Therefore, the demyelinated areas were identified based on their hypercellularity using DAPI staining for nuclei. Immunohistochemical analysis showed that fibronectin expression was highly detectable in demyelinated areas at 3 and 5 days post lesion, but progressively reduced at 10 and 15 days post lesion (Fig. 1B). Western blot analysis confirmed a clear and significant increase in fibronectin protein expression at 5 days post lesion, followed by a significant decrease at 14 days post lesion (Fig. 1C). Demyelination and ongoing remyelination of the lesions were confirmed by reduced expression levels of MBP at 5 days post lesion compared with unlesioned control, and increased MBP expression at 14 days post lesion as compared with 5

days post lesion, respectively (Fig. 1C). Transient expression of fibronectin was also observed using a second model of toxin-induced demyelination, in which ethidium bromide is injected into the caudal cerebral peduncle of rats (Supplementary Fig. 1; (Woodruff, Franklin 1999)).

Fibronectin has two major variants: (i) plasma fibronectin, a soluble dimer that is secreted into the circulation by hepatocytes; and (ii) cellular fibronectin, which is produced by resident cells. On CNS injury, and likely also on toxin-induced demyelination, plasma fibronectin enters the brain owing to blood–brain barrier disruption (Sobel, Mitchell 1989, van Horssen et al. 2005). To examine whether cells also express cellular fibronectin following CNS demyelination, we analysed fibronectin messenger RNA expression in lysolecithin-induced demyelination via reverse transcriptase–PCR. As shown in Fig. 1D, total fibronectin messenger RNA levels increased significantly at demyelination (5 days post lesion) compared with unlesioned spinal cord, and decreased again in early remyelination (14 days post lesion). To assess cellular fibronectin protein expression, we performed double immunohistochemistry with anti-fibronectin and anti-EIIIA (IST9) specific antibodies. EIIIA-fibronectin, i.e. cellular fibronectin, was expressed in acute lysolecithin-induced demyelination, where it was confined to the lesioned area and mostly located around blood vessels (Fig. 1E). Notably, fibronectin expression was more widespread throughout the demyelinated area than EIIIAfibronectin. Importantly, EIIIA-fibronectin protein expression was increased as early as 1 day post lesion and downregulated at 14 days post lesion (Supplementary Fig. 2), concomitant to the decrease of total fibronectin expression (Fig. 1B and C). Hence, these findings reveal that fibronectin expression was transiently upregulated on toxin-induced demyelination, and the detection of fibronectin messenger RNA and EIIIA-fibronectin indicate that CNS resident cells synthesize fibronectin in response to demyelination, which prompted us to identify these cells.

Multiple cell types express cellular fibronectin in toxin-induced demyelination

The cellular distribution of fibronectin messenger RNA in toxin-induced demyelination sections was examined by *in situ* hybridization. Fibronectin messenger RNA expression was confined to the demyelinated area (Fig. 2A), consistent with fibronectin protein expression (Fig. 1A). Macrophages/microglia were identified as a possible source by two-colour double labelling *in situ* hybridization for fibronectin and osteopontin messenger RNA, a marker for cells of the macrophage/microglia lineage particularly in toxin-induced lesions (Zhao et al. 2008). There was clear colocalization at 1 day post lesion (Fig. 2B; 68.2 ± 11.2%) that was decreased at 5 days post lesion (Fig. 2C; 23 ± 5.8%), which might reflect the transition from monocytes/microglia to phagocytotic macrophages (Zhao et al., 2006). This is supported by double labelling with anti-fibronectin and anti-CD11b (MAC1) antibodies, showing fibronectin expression in a substantial number of MAC1-positive microglia/macrophages (Fig. 2D, 5 days post lesion). Fibronectin messenger RNA (Fig. 2E) and protein (Fig. 2F) was further detected in GFAP-positive astrocytes, indicating that astrocytes are another source of cellular fibronectin. Co-labelling with anti-Olig2 antibodies (Fig. 2G; 2.9 ± 1.0% fibronectin messenger RNA/Olig2 double positive), as a marker for the oligodendrocytes lineage and antibodies against

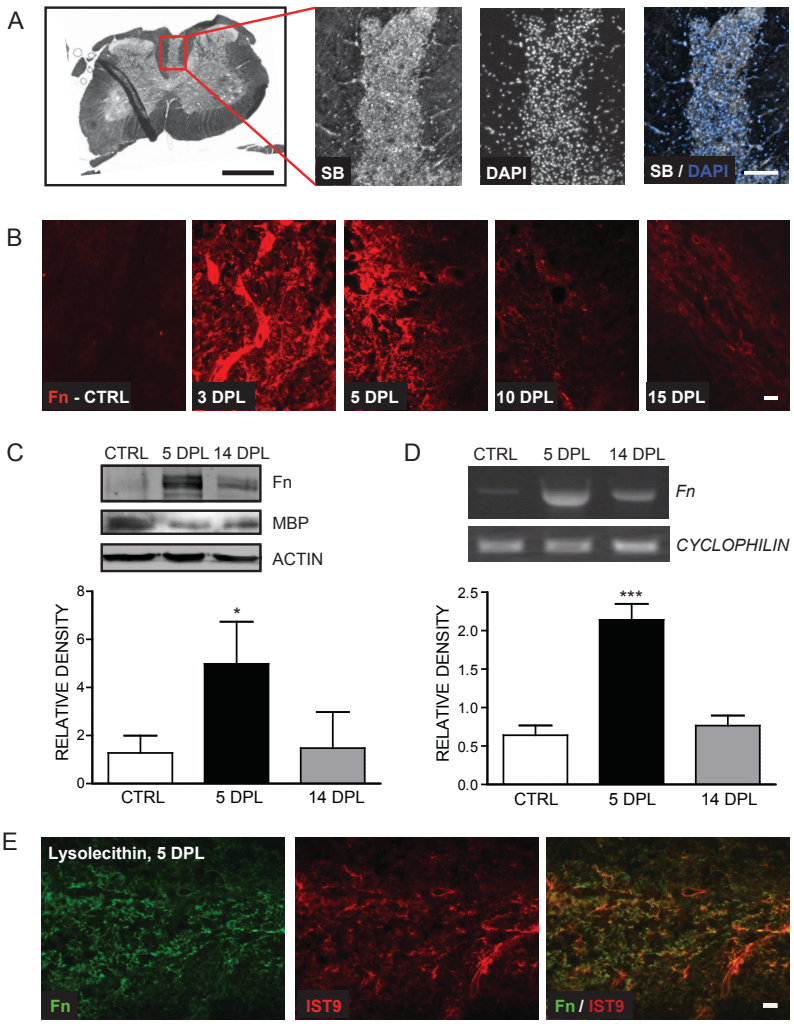


Figure 1. Expression of cellular fibronectin in lysolecithin-induced demyelination. Lesioned and non-lesioned tissues were analyzed for the expression of Fn protein and mRNA using fluorescent immunohistochemistry (B, E), Western blot (C) and RT-PCR (D) analysis. Focal demyelination of the rat spinal cord was induced by lysolecithin injection. In A-E, representative images, blots and gels are shown of 3-4 animals per condition. A. Demyelinated areas, as visualized with a Sudan black myelin stain, were identified based on their hypercellularity (DAPI staining). Images were taken at 5 days post lesion (DPL). Scale bars are 1000 µm (black) and 100 µm (white), respectively. B. Fn expression (red) is abundant at demyelination (3 and 5 DPL, and cleared upon remyelination (10 and 15 DPL). The baseline expression of Fn was very low upon immunostaining of unlesioned control spinal cord and normal appearing tissue around lesions. Scale bar is 20 µm. C, D. Fn protein (C) and mRNA (D) is upregulated in demyelination (5 DPL vs CTRL), but downregulated upon remyelination (14 DPL vs 5 DPL). Data were quantified by normalizing the optical densities of Fn protein against actin (C), and Fn mRNA against the housekeeping gene cyclophilin (D). Data are expressed as value of the mean + the standard deviation (* $p<0.05$, *** $p<0.0001$ at one-way ANOVA followed by Tukey's HSD test, 3-4 animals per condition). Demyelination of the lesions was confirmed by lower expression levels of the myelin protein MBP. E. Fn (red) and EIIIA-Fn (IST9, green) expression in lysolecithin-induced demyelination (5 DPL). Scale bar is 20 µm. EIIIA-Fn and Fn particularly co-localize (yellow) around blood vessels.

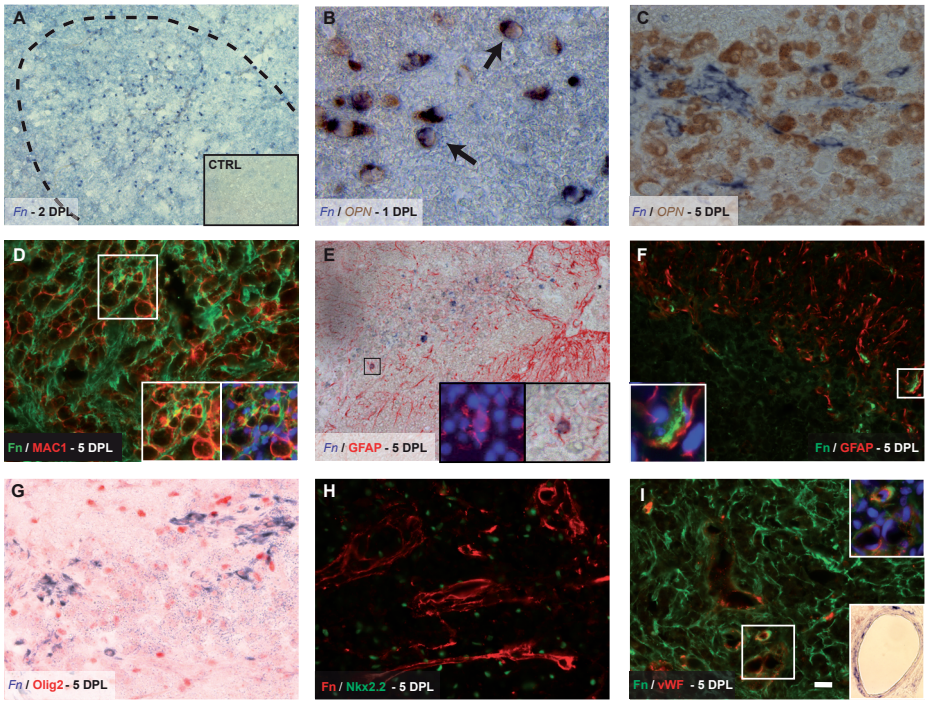


Figure 2. Expression of fibronectin by a variety of cell types in toxin-induced demyelination. Fn mRNA in lesioned areas is expressed in microglia/macrophages (B), astrocytes (E) and endothelial cells (inset I), but not oligodendrocytes (G). Sections from non-lesioned (inset A), CCP-lesions (A, E, F) and spinal cord lesions (lysolecithin, B-D, G-I) at 1 DPL (B), 2 DPL (A), and 5 DPL (C-I) were single (A, inset A and I) or double (B-I) labeled for either Fn protein (green, D, F, I and red, H), or Fn mRNA (A-C, E, G, inset I, blue) together with OPN mRNA (B, C, magenta) and MAC1 (D, red) as markers for microglia/macrophages, GFAP (D, E, red), as a marker for astrocytes, Olig-2 (G, red) as a marker for the oligodendrocyte lineage, Nkx2.2 (H, green) as a marker for OPCs, and Von Willebrand factor (vWF, I, red), as a marker for endothelial cells. Protein and mRNA were visualized by indirect fluorescent immunohistochemistry and in situ hybridization, respectively. Lesioned areas were identified by hypercellularity, i.e., DAPI (blue) staining. Representative images of 3-4 animals per condition are shown. DPL is days post lesion. Scale bar is 50 µm for A, E and H, and 20 µm for B-D, F, G and I.

Nkx2.2 (Fig. 2H), as a marker for oligodendrocyte progenitor cells, demonstrated little colocalization with fibronectin messenger RNA and protein, respectively. Finally, some fibronectin messenger RNA–expressing cells occurred in association with blood vessels and had the morphology and arrangement of endothelial cells (Fig. 2I, inset). Double labelling with antibodies against fibronectin and von Willebrand factor, a marker for endothelial cells, showed that some fibronectin co-localized with oval-shaped von Willebrand factor-positive cells (Fig. 2I, inset). This indicated that endothelial cells may also produce cellular fibronectin, as suggested before by the localization of EIIIA-fibronectin (Fig. 1), and co-localization of IST9 with von Willebrand factor (data not shown). Hence, macrophages/ microglia, astrocytes and endothelial cells, but not cells of the oligodendrocytes lineage, contribute to cellular fibronectin expression in demyelinated lesions.

Fibronectin assembles into aggregates in demyelinated multiple sclerosis lesions

Previous studies suggest the expression of fibronectin in chronically demyelinated multiple sclerosis lesions (Sobel, Mitchell 1989, van Horssen et al. 2005, Satoh, Tabunoki & Yamamura 2009). However, these studies do not provide information on fibronectin levels, its origin or its form. We therefore biochemically characterized fibronectin expression in white matter brain homogenates of healthy subjects (control white matter, n = 9), (chronic) active multiple sclerosis lesions (n = 8) and chronic inactive multiple sclerosis lesions (n = 9). In chronic inactive lesions, fibronectin was expressed around blood vessels (Fig. 3D), whereas an increased number of smaller fibronectin deposits were present throughout (chronic) active multiple sclerosis lesions (Fig. 3B and C). However, in control white matter, obtained from subjects without clinical or pathological signs of neurological disease, fibronectin was scarcely detectable and limited to the vasculature (Fig. 3A). Western blot analysis under reducing conditions confirmed the increased fibronectin expression in multiple sclerosis lesions as compared with control white matter (Fig. 3E). Although control white matter showed a variation in fibronectin levels among individual subjects, the amount of fibronectin in white matter homogenates of active and inactive multiple sclerosis lesions was generally higher (Fig. 3F). Therefore, our data provide quantitative evidence of fibronectin accumulation in multiple sclerosis lesions. Analysis of fibronectin messenger RNA expression via reverse transcriptase–PCR revealed low levels (Supplementary Fig. 3) that do not seem to be in line with the increased fibronectin protein levels in multiple sclerosis lesions (Fig. 3B), but could well be explained by the chronic status of these lesions. Accordingly, fibronectin messenger RNA might have been initially upregulated at the onset of demyelination, but downregulated over time, whereas the protein has not been degraded and subjected to post-transcriptional and post-translational modification, which we examined next.

Fibronectin normally appears as a disulphide-bound dimer with subunits of 220–250 kDa, but can assemble into a complex network of fibrils of high molecular weight aggregates, which are insoluble in the detergent deoxycholate (Wierzbicka-Patynowski, Schwarzbauer 2003, Mao, Schwarzbauer 2005). Under non-reducing SDS-PAGE conditions, fibronectin aggregates remain either in the stacking gel or just penetrate the resolving gel in lower percentage SDS cells. To examine whether fibronectin aggregates in multiple sclerosis lesions, we performed detergent deoxycholate (in)solubility assays. High molecular weight fibronectin complexes were observed in the deoxycholate-insoluble fraction from homogenates of multiple sclerosis lesions, whereas fibronectin dimers were the main form in control white matter (Fig. 3G). This suggests that fibronectin aggregates are predominantly present in both active and inactive multiple sclerosis lesions, and hardly in control white matter. Given our previous findings that fibronectin perturbs myelin-like membrane formation (Buttery, ffrench-Constant 1999, Siskova et al. 2006, Maier et al. 2005, Siskova et al. 2009) and given that clearance of fibronectin in toxin-induced demyelination preceded remyelination (Fig. 1), the detection of fibronectin aggregates in chronically demyelinated multiple sclerosis lesions led us to hypothesize that they may contribute to remyelination failure. If this is the case, fibronectin would not aggregate and/or aggregation would not persist in toxin-induced demyelination.

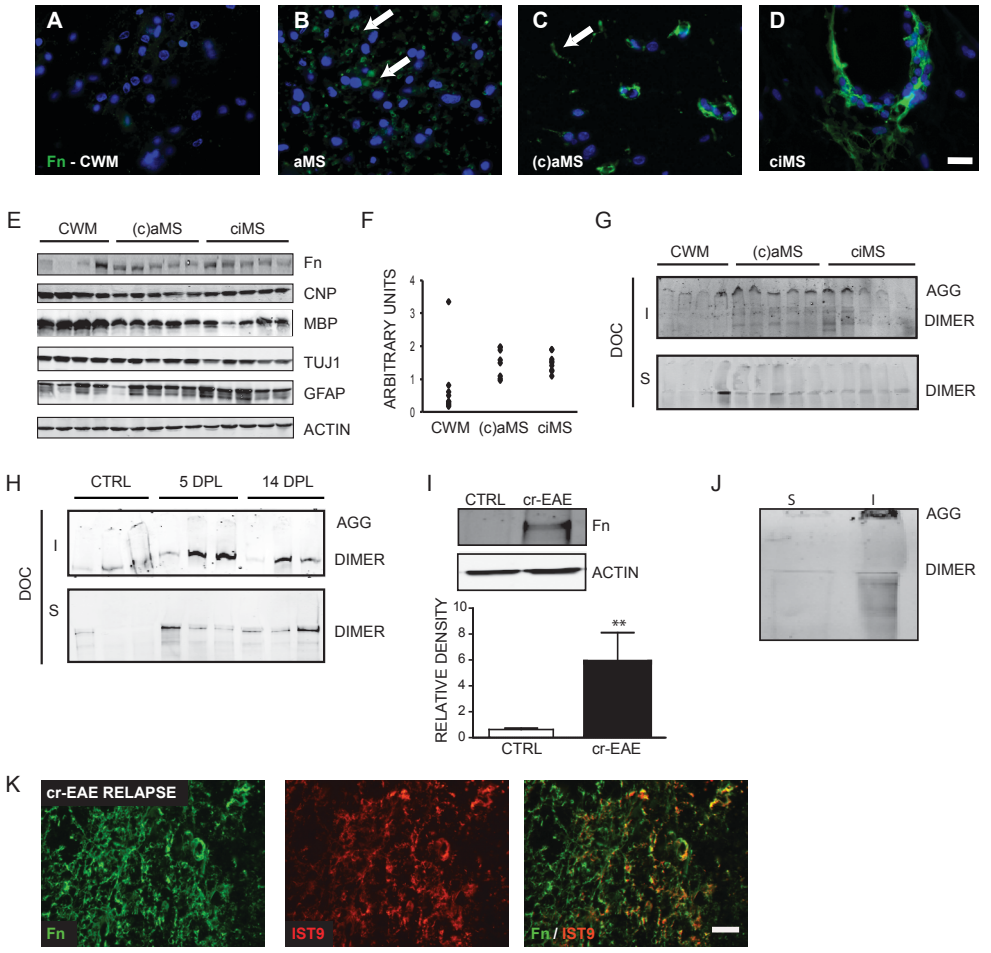


Figure 3. Fibronectin protein expression and biochemical characterization in human CWM, MS lesions, cr-EAE and toxin-induced lesions. A-D. Fn expression (green) is abundant within the parenchyma of demyelinated MS lesion areas in aMS (B, arrows) and caMS (arrow), and nearly absent from control white matter (CWM). In ciMS, Fn is abundantly expressed in association with blood vessels (D). Nuclei were counterstained with DAPI (blue). Representative images of CWM (A,) and centers of active (aMS, B), chronic active (caMS, C) and chronic inactive (ciMS, D) MS lesions are shown. Scale bar is 20 µm. E. Expression levels of Fn and the other indicated proteins in 50 µg of brain white matter homogenates of human CWM (4), (c) aMS (5) and ciMS (5) lesions as determined by Western blot under reducing conditions (monomers). Demyelination of the lesions was confirmed by lower expression levels of the myelin protein MBP compared to CWM. Decreased CNP-levels, mostly in active MS lesions, and decreased TuJ1-levels, particularly in inactive lesions, were likely indicative of OLG and axonal loss, respectively. The constant levels of actin in the samples served as internal controls. F. Variation of Fn expression in individual human white matter tissue. G. Fn aggregates (Fn AGG) are abundantly present in (c)aMS and ciMS lesions homogenates as compared to CWM homogenates. 100 µg of homogenates were subjected to DOC-(in)solubility assays, and DOC-insoluble (I) and -soluble (S) fractions were analyzed by Western blotting under non-reducing conditions. H. Fn aggregates are virtually absent from lysolecithin-induced demyelinated lesions. DOC-insoluble (I) and -soluble (S) fractions from 50 µg tissue were analyzed by Western blotting under non-reducing conditions. 3 animals per condition were analyzed. (Legends continue on the next page.)

Fig. 3 continued. Fibronectin protein expression and biochemical characterization in human CWM, MS lesions, cr-EAE and toxin-induced lesions. (...) I. Fn expression is upregulated in cr-EAE animals as compared to IFA controls. Spinal cord homogenates (20 µg) of control and cr-EAE (relapse phase) were analyzed by Western blotting under reducing conditions (monomers). Data were quantified by normalizing the optical densities of Fn protein against actin, and expressed as mean + the standard deviation (** $p < 0,01$ at Student's t-test, 3 animals per group). J. Fn aggregates at the relapse phase in cr-EAE. DOC-insoluble (I) and -soluble (S) fractions from 100 µg tissue were analyzed by Western blotting under non-reducing conditions. K. Fn (red) and EIIIA-Fn (IST9, green) expression at the relapse phase of cr-EAE. Scale bar is 20 µm. EIIIA-Fn and Fn particularly co-localize (yellow) around blood vessels.

Fibronectin aggregates predominantly in inflammation-induced demyelination

Deoxycholate (in)solubility assays followed by SDS-PAGE under non-reducing conditions revealed that fibronectin aggregation was minimal in toxin-lesioned tissues (Fig. 3H). Hence, fibronectin expression was transiently upregulated on toxin-induced demyelination, but does not extensively aggregate. A major difference between multiple sclerosis and toxin-induced lesions is the mechanism of demyelination. In multiple sclerosis, demyelination results mainly from activity of the adaptive immune system, whereas in toxin-induced models, demyelination is caused by the toxin and this initiates a secondary inflammatory response, mainly involving the innate immune system. To assess whether fibronectin aggregation is mediated by chronic inflammation, we next examined fibronectin expression and aggregation in chronic relapsing EAE, an animal model of immune-mediated demyelination that resembles many clinical and pathological features of relapsing-remitting multiple sclerosis (Furlan, Cuomo & Martino 2009, Pachner 2011). On immunization with a myelin oligodendrocyte glycoprotein peptide, the animals suffer from neurological symptoms, usually restricted to the hind body, that develop in a relapsing-remitting pattern (Supplementary Fig. 4A). During the relapse phase, Major Histocompatibility Complex class II inflammatory infiltrates can be observed in the rat spinal cord (Supplementary Fig. 4B), whereas demyelinated areas can be observed in the lumbar and sacral region of the spinal cord (Ledeboer et al. 2003, Storch et al. 1998). Total fibronectin expression was increased at the relapse phase in chronic relapsing EAE by ~9-fold (Fig. 3I) compared with incomplete Freund's adjuvant control. Furthermore, deoxycholate-(in)solubility assays showed that fibronectin aggregated in chronic relapsing EAE (Fig. 3J), similar to multiple sclerosis lesions (Fig. 3B). EIIIA-fibronectin expression was also evident at the relapse in chronic relapsing EAE (Fig. 3K), suggesting that cellular fibronectin was increased as an immediate response on inflammation-mediated demyelination. Hence, whereas fibronectin was transiently expressed and remained predominantly soluble in toxin-induced demyelination undergoing remyelination, fibronectin aggregates were formed in lesions of inflammation-induced demyelination, where remyelination often fails. Therefore, we next examined whether inflammatory mediators induce fibronectin aggregation *in vitro*.

Fibronectin aggregation by cultured astrocytes is induced by the inflammatory mediator lipopolysaccharide

Having identified multiple cellular sources *in vivo*, we first examined which of these cells express fibronectin messenger RNA and protein in (primary) monoculture *in vitro*. Only cultured astrocytes produced fibronectin messenger RNA and fibronectin protein in considerable amounts (Fig. 4A), which corroborate previous findings that astrocytes produce fibronectin (Price, Hynes 1985, Liesi, Kirkwood & Vaheri 1986, Oh, Yong 1996). However, fibronectin expression by the other cell types may occur in other, particular activation states. Water-lysis of the cultured astrocytes showed that untreated astrocytes deposited fibronectin in an evenly distributed diffuse pattern, whereas astrocytes exposed to the inflammatory mediator lipopolysaccharide deposited a more localized fibrillar fibronectin matrix (Fig. 4B, rat). Similar results were obtained when astrocytes were removed by EDTA-mediated detachment (data not shown). Notably, an increase in fibronectin messenger RNA and a slight increase in protein expression were observed in astrocytes treated with the inflammatory mediator lipopolysaccharide (Fig. 4A). Fibronectin aggregation also markedly increased after treatment with lipopolysaccharide in ratastrocyte-derived matrices, as detected by deoxycholate-(in)solubility assays (Fig. 4C). Likewise, cultured human astrocytes isolated from patients with multiple sclerosis displayed the ability to deposit and to arrange fibronectin in fibrillar networks, whereas human astrocytes from healthy subjects deposited fibronectin in smaller more diffuse structures (Fig. 4B, human). In addition, astrocytes from multiple sclerosis tissue synthesized slightly more fibronectin and similar levels of laminin, which is a myelination permissive extracellular matrix molecule (Buttery, ffrench-Constant 1999, Siskova et al. 2006, Colognato, ffrench-Constant & Feltri 2005) compared with astrocytes from healthy subjects (Fig. 4D). Furthermore, multiple sclerosis astroglial matrices, but not those from healthy astrocytes, showed a clear band at the position of aggregated fibronectin in the deoxycholate-insoluble fraction (Fig. 4E), whereas deposited laminin appeared in the deoxycholate-soluble fraction (Fig. 4E). Also, preliminary proteomic analysis demonstrated that the deoxycholate-insoluble fraction mainly consists of fibronectin, i.e. ~10% of total protein, and does not contain other extracellular matrix proteins, such as hyaluronan and proteoglycans. These findings demonstrate that, although cultured astrocytes synthesize and deposit fibronectin, they do not induce aggregation unless additional factors, such as those linked to chronic inflammation, are present. This could explain why fibronectin aggregates were found at the relapse phase in chronic relapsing EAE and multiple sclerosis lesions, but not in toxin-induced demyelination. Because fibronectin aggregates in multiple sclerosis lesions, where remyelination fails, and not in toxin-induced lesions, where remyelination is complete, we next examined whether fibronectin aggregates contribute directly to remyelination failure.

Astrocyte-derived fibronectin aggregates inhibit remyelination

We first studied whether astrocyte-derived fibronectin aggregates altered progression within the oligodendrocytes lineage *in vitro*. To allow investigation of cell–matrix interactions as such,

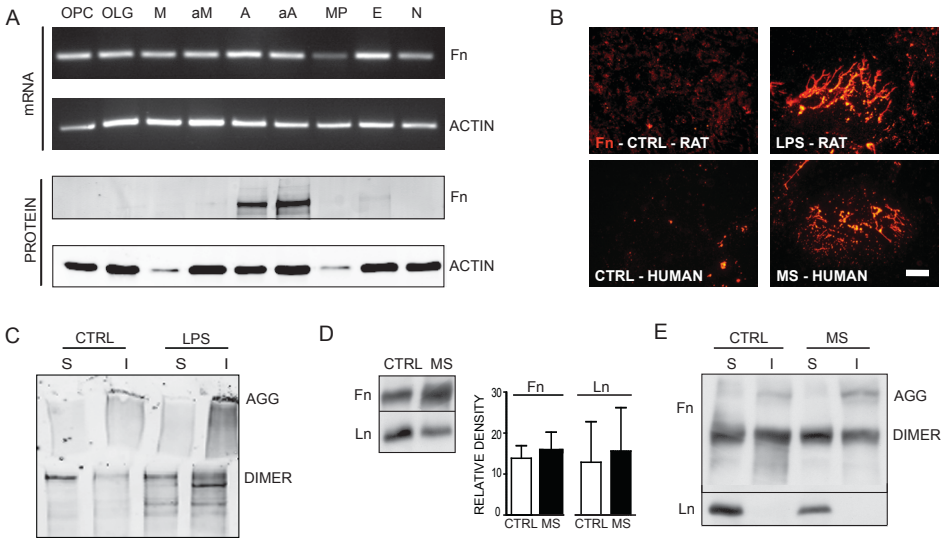
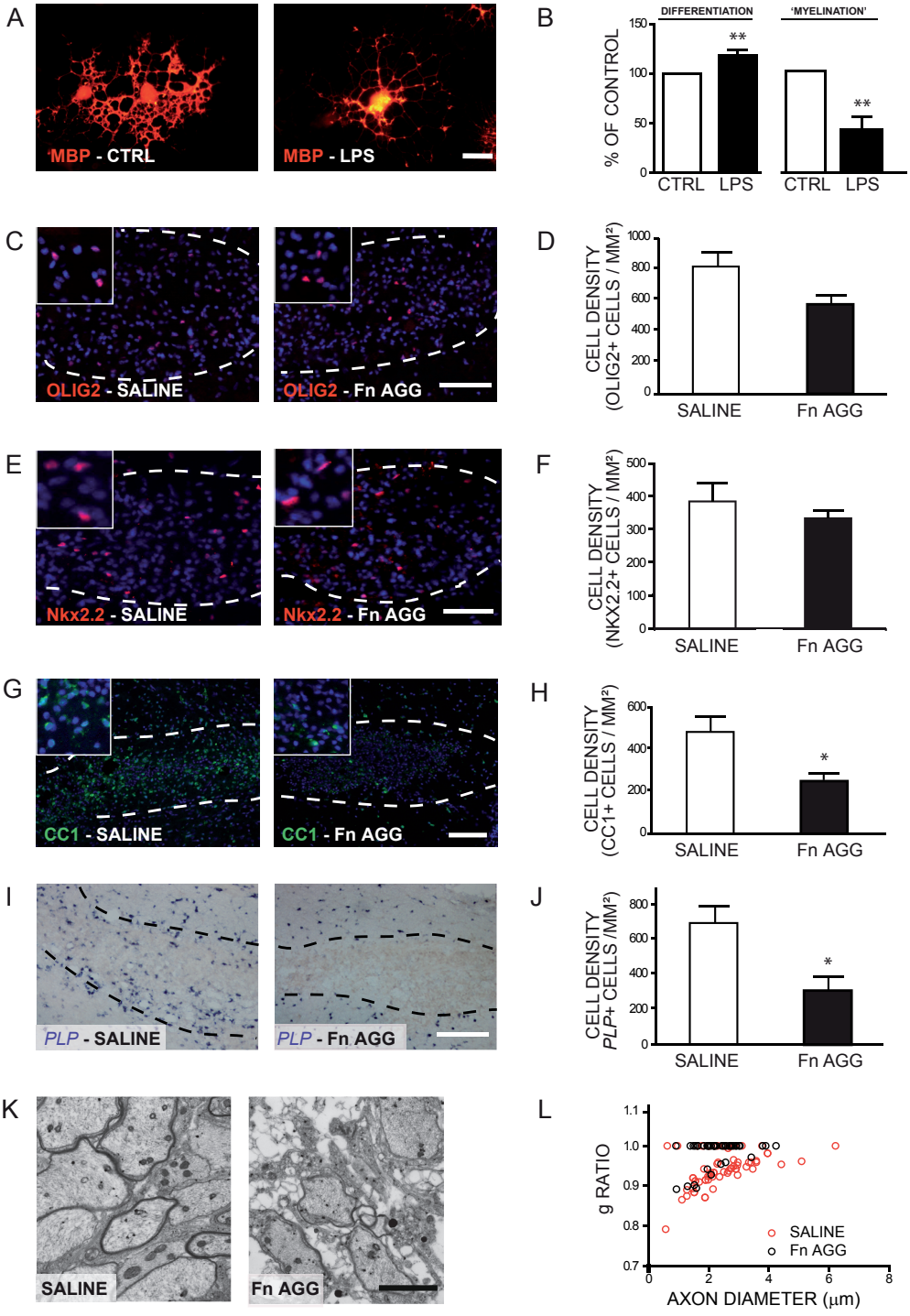


Figure 4. Fibronectin expression and aggregation in cultured cells. A. Cultured astrocytes synthesize Fn protein in abundant levels. Primary rat oligodendrocyte progenitors (OPC), mature rat oligodendrocytes (OLG), rat microglia (M), rat LPS-activated microglia (aM), rat astrocytes (A), rat LPS-activated astrocytes (aA), bone-marrow derived macrophages (MP), rat cortical neurons (N) (de Jong et al., 2005), and the human brain endothelial cell line hCEMC/D3 (E) (Weksler et al. 2005) were analyzed for Fn mRNA and protein expression using RT-PCR and Western blot analysis (reducing conditions, monomers). A representative blot of 3 independent experiments is shown. B. Astroglial matrices from both LPS-activated rat astrocytes (LPS) and human MS astrocytes (MS) contained fibrillar Fn structures, whereas control astrocytes (CTRL) showed a more diffuse deposition of Fn. Cultured astrocytes were water-lysed and remaining deposits were analyzed for Fn deposition by immunofluorescence. Scale bar is 20 µm. Representative images of 3-4 independent experiments are shown. C. Fn aggregates (Fn AGG) are present in the matrix of LPS-stimulated (+ LPS), but not in the matrix of non-stimulated (-) rat astrocytes. 10 µg deposits were subjected to DOC-(in)solubility assays, and DOC-insoluble (I) and -soluble (S) fractions were analyzed by Western blotting under non-reducing conditions. Representative blots and images of 3-4 independent experiments are shown. D. Fn and laminin (Ln) are expressed by cultured human astrocytes from healthy subjects (CTRL, 4) and MS patients (3). Total cell lysates (10 µg) were analyzed for Fn and Ln expression by Western blotting under reducing conditions (monomers). Data were quantified by normalizing the optical densities of Fn and Ln protein against actin, and expressed as mean + the standard deviation ($p > 0,05$ at Student´s t-test). E. Presence of Fn aggregates is increased in the DOC-insoluble fraction of MS astrocytes (MS) as compared to normal astrocytes (CTRL), whereas laminin is DOC-soluble. 10 µg deposits were subjected to DOC-(in)solubility assays, and DOC-insoluble (I) and -soluble (S) fractions were analyzed by Western blotting under non-reducing conditions. Representative blots of the deposits of astrocyte cultures analyzed in D are shown.

oligodendrocyte progenitor cells were plated onto astroglial matrices obtained from untreated (Fig. 4B) or lipopolysaccharide-treated rat astrocytes (Fig. 4B). The astrocytes were removed by water-lysis, so that the effect of live astrocytes and their soluble factors on oligodendrocyte progenitor cells was eliminated. When oligodendrocyte progenitor cells were cultured on the astrocytederived matrices for 7 days, a small increase in the number of MBP-positive cells, indicative for differentiation, was observed (Fig. 5A and B). Myelin-like membrane formation, as defined by cells bearing MBP-

positive membranous structures spread between the cellular processes, however, was prevented on astroglial matrices from lipopolysaccharide-stimulated astrocytes, i.e. containing fibronectin aggregates, compared with oligodendrocyte progenitor cells cultured on astroglial matrices from untreated rat astrocytes (Fig. 5A and B). To further examine whether fibronectin aggregates in the total astroglial matrix indeed impair remyelination *in vivo*, enriched fibronectin aggregates, obtained from the deoxycholate-insoluble fraction of deposits from cultured rat astrocytes, were intralesionally injected in lysolecithin-induced demyelinated lesions at 7 days post lesion. Following fibronectin aggregate injection, the density of cells expressing the oligodendrocytes marker Olig2 seemed slightly reduced at 14 days post lesion, i.e. 7 days post aggregate injection, compared with saline-injected lesions (Fig. 5C and D), though not statistically significant. Notably, in saline-injected lesions, substantial fibronectin staining was still present, which is likely due to the response of fresh trauma by the (second) injection, although to a much lesser extent comparing with fibronectin aggregate–injected esions (Supplementary Fig. 5A and B). The number of cells expressing the oligodendrocyte progenitor cell marker Nkx2.2 in the lesions was also not altered by fibronectin aggregates (Fig. E and F). Cells expressing oligodendrocyte differentiation markers APC protein (CC1) and proteolipid protein messenger RNA were, however, significantly decreased (Fig. 5G–J). Electron microscopy analysis at 14 days post lesion revealed that remyelination was impaired on fibronectin aggregate injection (Fig. 5K), which was reflected by a significant increase in the g-ratios (P50.05, Fig. 5L). To examine whether the reduction of mature oligodendrocytes was due to direct toxicity of fibronectin aggregates, the viability of oligodendrocyte cell lineage cells was assessed *in vitro* with

Figure 5. Effects of fibronectin aggregates on oligodendrocytes *in vitro* and *in vivo*. A, B OPCs differentiate to MBP-expressing oligodendrocytes on control (ECM CTRL) and Fn aggregate (ECM LPS) astroglial matrices, but myelin-like membrane formation was retarded on Fn aggregates (Fn AGG). This is confirmed by quantification of MBP-positive cells (B, differentiation), and the amount of MBP-positive cells elaborating myelin-like membranes (B, ´myelination´). Astroglial matrices were obtained by water-lysis of astrocytes. In each experiment, at least 500 cell/wells were counted. To compare different independent experiments, the data are expressed as % of control, i.e., values obtained from CTRL were set to 100. Each bar represents the mean + SEM of 3 independent experiments. Statistical differences were assessed with a Student´s t-test, and are indicated by asterisks (* p <0.05, ** p <0.01). Representative images of 3 independent experiments are shown (A). Scale bar is 20 µm. C-L. Fn aggregates impair OLG differentiation and remyelination in vivo. DOC-insoluble Fn aggregates obtained from rat astrocyte-derived deposits were intralesionally injected into demyelinated lesions of rats, 7 days after lysolecithin-induced demyelination, and the analyzed at 14 DPL, i.e., 7 days post injection. C-J. Images in C, E, G and I are representative images of demyelinated areas upon saline (left) and Fn aggregate injection. The outline of the lesions was measured using AxoVision software on the basis of the increase in DAPI staining. The numbers of Olig2 (C), NKX2.2 (E), CC1 (G), or PLP mRNA (I) positive cells were manually counted 3 times. There are 3-4 animals per group, 3 lesions per animal at a distance of about 120 µM apart from each other. The quantifications are shown in D, F, H and J, respectively. Each bar represents the mean + SEM. Statistical differences were assessed with a Student's t-test, and are indicated by asterisks (* p <0.05). Scale bar is 100 µm. K, L. Images are representative electromicrographs of demyelinated areas upon saline (left) and Fn aggregate injection. The g-ratio of individual axons from saline injected (red circles) and Fn aggregate (black circles) were plotted against the corresponding axon diameters. The average g-ratio in Fn aggregated injected demyelinated areas is significantly higher than upon saline injection (p<0.05, Student´s t-test, 3 animals per group). Scale bar in K is 2 µm.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a lactate dehydrogenase and MTT assay. Oligodendrocyte viability was not affected by addition of fibronectin aggregates to different maturation stages (Supplementary Fig. 5C and D), indicating that fibronectin aggregates are apparently not toxic to cultured oligodendrocytes. Therefore, these data indicate that astrocytederived fibronectin aggregates predominantly perturbed oligodendrocytes differentiation *in vivo* and impaired remyelination.

These findings would imply that fibronectin aggregates must be absent from remyelinated multiple sclerosis lesions, i.e. shadow plaques, which were examined next.

Fibronectin aggregates are present at low level in remyelinated multiple sclerosis lesions

To assess whether fibronectin aggregates are expressed at remyelinating conditions, homogenates of remyelinated multiple sclerosis shadow plaque containing brain tissue were subjected to deoxycholate-(in)solubility assays. The status of shadow plaques was confirmed by the Netherlands Brain Bank, showing a slightly reduced expression of proteolipid protein compared with the surrounding normal-appearing white matter, without abundant expression of CD68. Fibronectin aggregates were weakly expressed in the examined multiple sclerosis shadow plaques, whereas fibronectin is mainly present as a deoxycholate-soluble dimer (Fig. 6A). Furthermore, western blot analysis at reducing conditions revealed that fibronectin levels are slightly increased in remyelinated multiple sclerosis lesions compared with control white matter, but to a lesser extent than in (chronic) active multiple sclerosis lesions (Fig. 6B). Fibronectin staining around blood vessels can be discerned throughout the shadow plaques (Fig. 6C), and seemed higher than fibronectin staining in surrounding normal-appearing white matter (data not shown). However, fibronectin is barely detectable in the parenchyma of the emyelinated area. Thus, fibronectin aggregates are present at a low level in remyelinated multiple sclerosis lesions, which corroborate the findings that fibronectin aggregates impair remyelination.

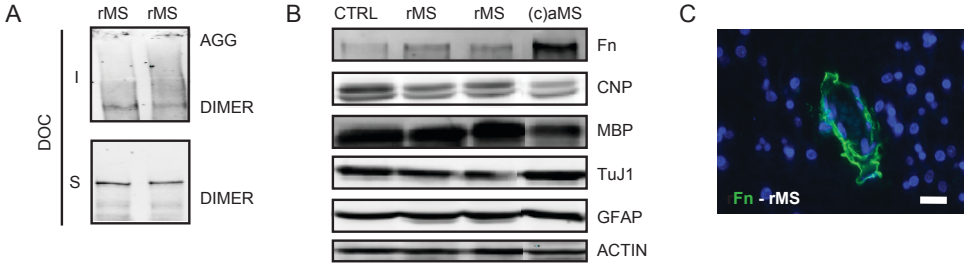

Figure 6. Fibronectin protein expression and biochemical characterization in remyelinated MS lesions.
A. Expression levels of Fn aggregates (Fn AGG) and dimers in remyelinated MS lesions (rMS). 100 µg of homogenates were subjected to DOC-(in)solubility assays, and DOC-insoluble (I) and -soluble (S) fractions were analyzed by Western blotting under non-reducing conditions. B. Total Fn expression in remyelinated MS lesions (rMS), as compared to control white matter (CWM), and (chronic) active MS [(c)aMS]. Expression levels of Fn and the other indicated proteins were detected by subjecting 50 µg of human brain white matter homogenates to Western blot analysis under reducing conditions. D. Immunohistochemistry for Fn in MS shadow plaque lesions, where Fn was detected in a shape typical for blood vessels. A representative image of two different remyelinated lesions is shown. Scale bar is 20 µm.

DISCUSSION

Remyelination is crucial for functional recovery in demyelinating diseases, such as multiple sclerosis (Duncan et al. 2009). Here, we show that the extracellular matrix component fibronectin, which is largely absent from healthy adult white matter, rapidly accumulates as an acute response to demyelination, but disappears on remyelination. However, in multiple sclerosis lesions where remyelination fails, a persistent accumulation of stable aggregated fibronectin occurs. These fibronectin aggregates are most likely generated by astrocytes on engagement with inflammatory mediators, and most relevantly, these aggregates impair remyelination.

Aggregation of fibronectin is likely associated with the process of inflammation. This was suggested by the absence of fibronectin aggregates from toxin-induced demyelination, where an innate immune response is secondary to demyelination, compared with the abundance of fibronectin aggregates in chronic relapsing EAE and multiple sclerosis lesions, where demyelination is caused by the adaptive immune response. Also, our *in vitro* experiments with primary rat astrocytes suggest a role for inflammatory mediators in fibronectin aggregation. Astrocytes exposed to the inflammatory mediator lipopolysaccharide deposit fibronectin in fibril-like structures, whereas untreated astrocytes deposit fibronectin in a more diffuse pattern. This structural difference in fibronectin appearance is monitored by conversion from a deoxycholate-soluble dimeric form to deoxycholate-insoluble fibronectin aggregates. Fibrillar fibronectin assembly is primarily initiated by binding of soluble fibronectin dimers to integrin α5β1, followed by assembly into high molecular weight fibronectin that is cross-linked by non-covalent bonds (Wierzbicka-Patynowski, Schwarzbauer 2003, Wu et al. 1993, Wu et al. 1995, Ohashi, Erickson 2009). Integrin α5β1 is expressed on astrocytes (King, McBride & Priestley 2001), neurons (King, McBride & Priestley 2001) and activated microglia (Milner et al. 2007), but not on oligodendrocytes (Milner, ffrench-Constant 1994). In addition to fibril formation, fibronectin can be covalently cross-linked by enzymes. Tumor necrosis factor-α, for example, increases fibronectin aggregation in endothelial cell layers by an enhanced transglutaminase activity on their surface (Chen et al. 2000), a process that might be active in multiple sclerosis lesions (van Strien et al. 2011). Hence, both persistent and recurring inflammation as well as concomitant astrogliosis could gradually result in conformation and accumulation of aggregated fibronectin in multiple sclerosis lesions.

Fibronectin expression in multiple sclerosis lesions was previously suggested to result from blood–brain barrier disruption (Sobel, Mitchell 1989, van Horssen et al. 2005). Our data reveal that on CNS injury, CNS resident cells also contribute to the fibronectin pool. This conclusion is supported by at least three observations: (i) next to a typical vasculature ocalization, small fibronectin deposits are present in multiple sclerosis, chronic relapsing EAE and toxin-induced lesions, suggesting additional sources to plasma leakage; (ii) fibronectin messenger RNA is upregulated in toxininduced demyelination; and (iii) EIIIA-fibronectin protein is detected at the lesion site. The lack of fibronectin messenger RNA, likely due to the chronic disease status, precluded elucidation of cellular sources in multiple sclerosis lesions. However, *in situ* hybridization co-localization studies in toxin-induced

lesion revealed that astrocytes, microglia/macrophages and endothelial cells likely synthesize fibronectin. Whether these cells also secrete and actively deposit fibronectin *in vivo* is rather difficult to determine. *In vitro*, fibronectin aggregation is dependent on astrocytes, as only astrocytes actively synthesize and deposit fibronectin, and plasma fibronectin alone, i.e. in the absence of astrocytes, does not aggregate (our unpublished observations). However, plasma fibronectin likely incorporates into astrocyte-derived fibronectin aggregates (McKeown-Longo, Mosher 1983, Peters et al. 1990).

Importantly, our data revealed that aggregated fibronectin impairs remyelination. Fibronectin aggregates are hardly present in remyelinated multiple sclerosis shadow plaques, and injection of astrocyte-derived fibronectin aggregates into lysolecithin-induced demyelinated lesions resulted in a significant decrease of differentiated oligodendrocytes and concomitant increase in g-ratio compared with saline-injected lesions. Myelin-like membrane formation *in vitro* is inhibited by astrocyte-derived matrix that contains fibronectin aggregates, but not by astrocyte-derived matrix that contains dimeric fibronectin. This might be explained by the presence of additional (extracellular matrix) molecules that reside in the total astroglial matrix, such as laminin ((Liesi, Dahl & Vaheri 1983, Liesi et al. 1984); Fig. 4D and E). These additional molecules could overcome the myelination-inhibiting effect of dimeric fibronectin that has been observed previously (Buttery, ffrench-Constant 1999, Siskova et al. 2006, Maier et al. 2005, Siskova et al. 2009). Indeed, laminin signals are known to dominate over dimeric plasma fibronectin inhibitory signals (Buttery, ffrench-Constant 1999). The presence of additional proteins in total astrocyte deposits may therefore also explain why fibronectin aggregates affect oligodendrocyte differentiation only *in vivo*, as the intralesionally injected aggregates were deoxycholate-insoluble, therefore lacking laminin and other glycoproteins (Fig. 4D and E). The inhibitory effect of fibronectin aggregates may occur through different mechanisms. First, fibronectin aggregates could directly affect oligodendrocyte progenitor cells through the classical fibronectin receptors, αv integrins, which they upregulate in toxin-induced lesions (Zhao et al. 2009). Alternatively, the interaction of aggregates with other cell types *in vivo* might indirectly affect oligodendrocyte differentiation and remyelination. Finally, fibronectin aggregation likely involves biochemical restructuring of fibronectin (Johnson et al. 1999, Baneyx, Baugh & Vogel 2002), which could expose different conformation-dependent binding sites that provoke altered signalling properties (Morla, Zhang & Ruoslahti 1994, Pasqualini et al. 1996, Sottile, Hocking & Swiatek 1998).

In conclusion, myelin regeneration following demyelination is a dynamic process, and requires a spatial and timely balanced response of the extracellular microenvironment. Temporal dimeric fibronectin expression by astrocytes might be important in regulating remyelination at earlier stages. Pathological fibronectin aggregates as observed in multiple sclerosis lesions do, however, likely contribute to remyelination failure. Therefore, strategies to promote remyelination should not aim at preventing fibronectin deposition, but at interfering with fibronectin aggregation and clearance. In addition, the effects of persistent fibronectin aggregates on other CNS cells, including

microglia and neurons, as well as the mechanisms of how aggregation is mediated, warrant further investigation, particularly because protein aggregation is likely central to the pathology of several other neurodegenerative diseases (Jucker, Walker 2011).

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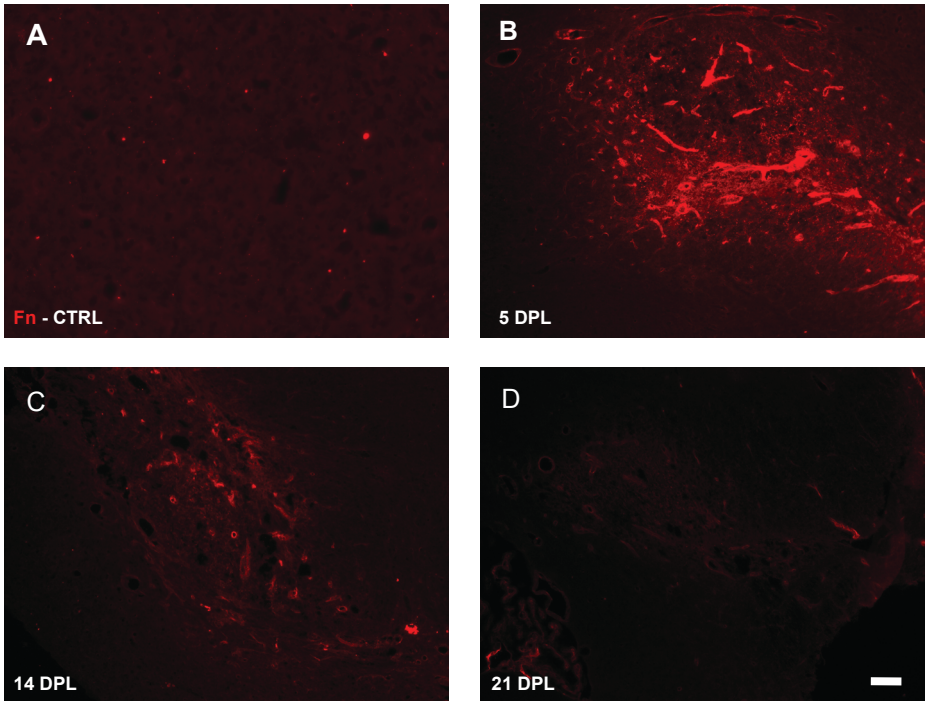
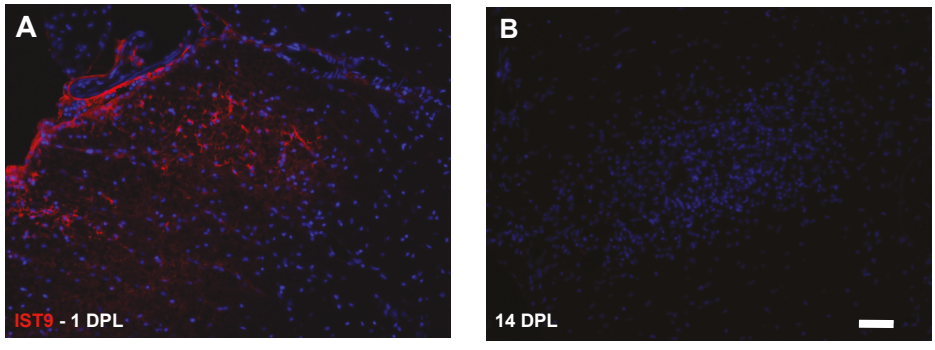
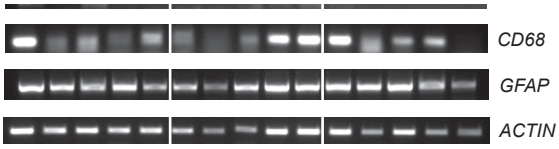


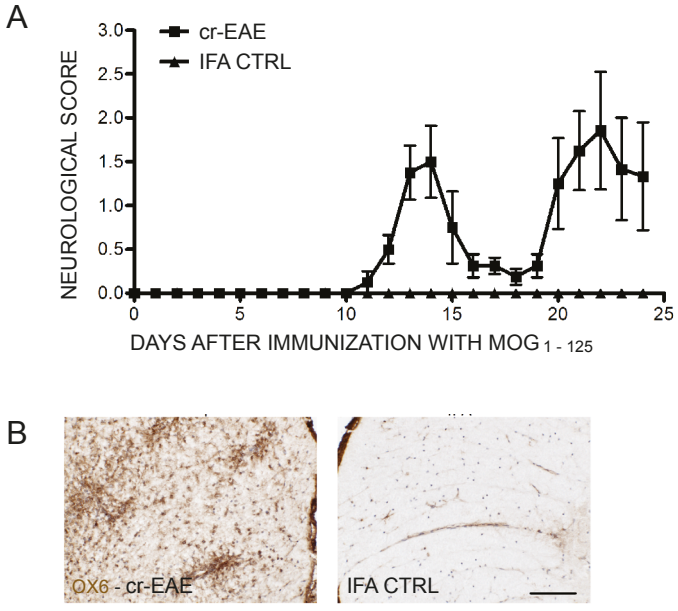
Supplementary Figure 1. Expression of fibronectin in caudal cerebral peduncle demyelinated lesions. A-D. Fibronectin (Fn) is abundantly expressed in demyelination (B,C, 5-14 DPL), and cleared upon remyelination (D, 21 DPL). Sections from non-lesioned (A, CTRL) and ethidium bromide-lesioned rat brain at different days post lesion (DPL) were immunostained for total Fn. In caudal cerebral peduncle demyelinated lesions, virtually no remyelination can be detected at 0-14 DPL (Zhao C, Li WW, Franklin RJM, 2006). Representative images of 3 animals per time points are shown. Scale bar is 20 µm.



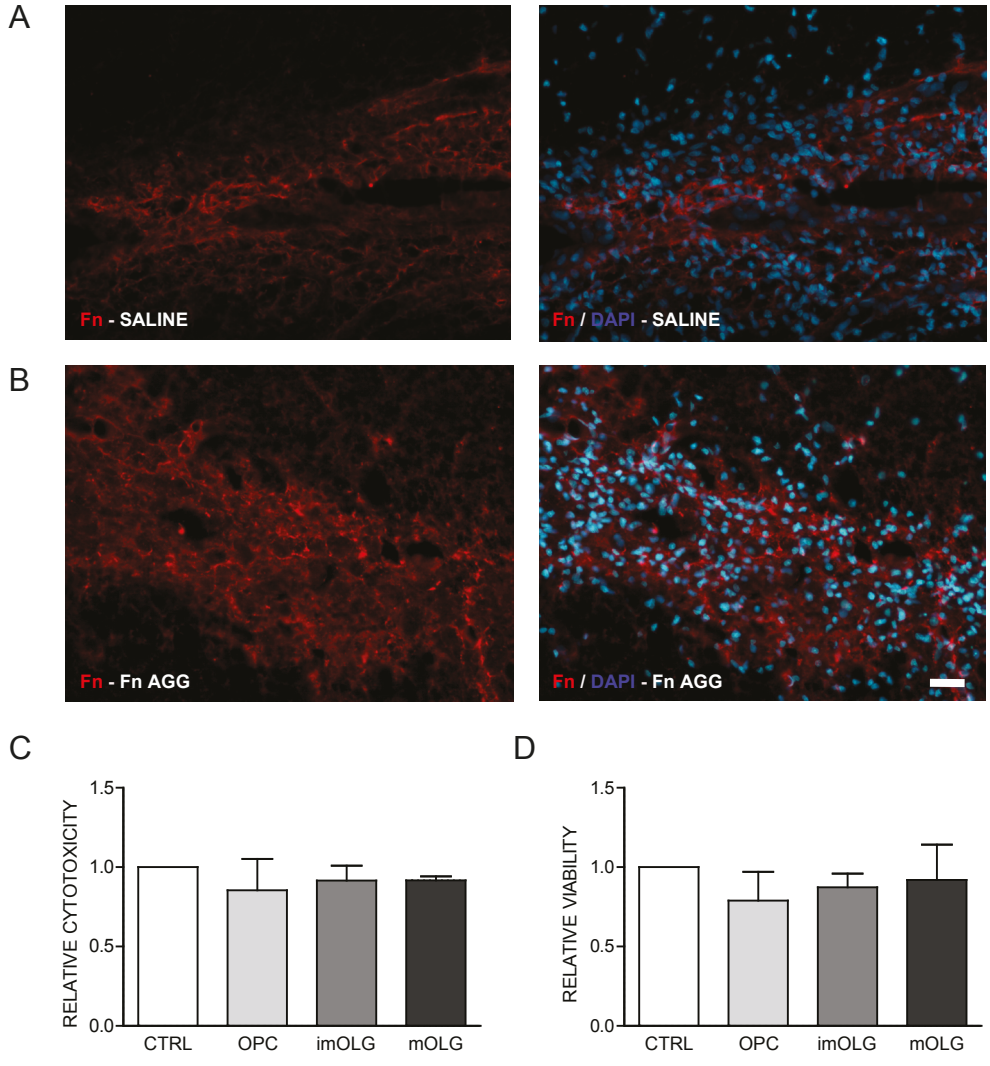
Supplementary Figure 2. Expression of EIIIA-fibronectin in lysolecithin-induced demyelinated lesions. EIIIA-fibronectin (EIIIA-Fn) is expressed as early as 1 day post lesion (DPL), and is cleared at 14 DPL. Sections of lysolecithin-induced lesioned areas at 1 (A) and 14 (B) DPL were immunostained for EIIIA-Fn (IST9, red). Representative images are shown. Scale bar is 20 µm.



Supplementary Figure 3. Fibronectin mRNA expression in human CWM and MS lesions. Fibronectin mRNA (Fn) is virtually absent from chronic active [(c)aMS] and chronic inactive (ciMS) MS lesions. Analysis of Fn, CD68 (activated microglia/macrophages), GFAP (astrocyte) mRNA levels (primers in table 1) in CWM, (c)aMS and ciMS homogenates. Actin mRNA served as an internal control. Two out of eight (chronic) active lesions showed Fn mRNA, whereas Fn mRNA was scarcely detectable in any of the seven chronic inactive MS lesions examind. Fn mRNA was also detected in three out of nine CWM tissues.



Supplementary Figure 4. Clinical scores and monocyte infiltration at the relapse phase of rat chronic experimental allergic encefalitis. A. Clinical scores of chronic experimental allergic encefalitis (cr-EAE) rats and incomplete Freund's adjuvant (IFA) controls. Cr-EAE animals were scored on the following scale:
0, no clinical disease; 0.5, partial loss of tail tone; 1, complete tail atony; 2, paresis, partial hind limb paralysis; 3, complete paralysis of the hind limbs and/or lower part of the body; 4, moribund or dead due to EAE. Data are represented as mean + S.E.M, with n=8 animals per group. B. Immunohistochemical staining for MHC class II positive macrophages (Ox6), showing a clear increase in cr-EAE animals as compared to IFA controls. Stainings were performed on the cervical part of the spinal cord from 3 representative animals, with average clinical scores during the relapse phase of cr-EAE.



Supplementary Figure 5. Fibronectin expression in lysolecithin lesions upon fibronectin aggregate injection, and assessment of the toxicity of fibronectin aggregates. Sections of lysolecithin-lesioned areas at 7 days post saline (A) or fibronectin aggregate (B) (Fn AGG) injection were immunostained for fibronectin (Fn). Representative images are shown. Scale bar is 20 µM. C, D. Fn aggregates were added to cultured rat oligodendrocytes at a final concentration of 5,0 µg/mL in various stages of oligodendrocyte maturation. Three days after Fn aggregate addition, LDH (C) and MTT (D) assays were performed. Relative cytotoxicity and viability were calculated against unstimulated control cells. Data are represented as the mean + the standard deviation. Statistical analyses were performed using one-way ANOVA.

Chapter 3

The EIIIA domain from astrocyte-derived fibronectin mediates proliferation of oligodendrocyte progenitor cells following CNS demyelination

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ABSTRACT

Central nervous system remyelination by oligodendrocyte progenitor cells (OPCs) ultimately fails in the majority of multiple sclerosis (MS) lesions. Remyelination failure is mediated in part by the aberrant expression of factors that impair OPC differentiation, including fibronectin (Fn) aggregates. Fn is present in demyelinated lesions in two major forms; plasma Fn (pFn), deposited following blood-brain barrier disruption, and cellular Fn (cFn), synthesized by resident glial cells and containing alternatively spliced domains EIIIA and EIIIB. Here, we investigated the distinctive roles that cFn and pFn play in remyelination. We used an inducible Cre-lox recombination strategy to selectively remove pFn, astrocyte-derived cFn or both from mice, and examined the impact on remyelination of toxin-induced demyelinated lesions of spinal cord white matter. This approach revealed that cFn from astrocytes is a major source of Fn in demyelinated lesions. Furthermore, following astrocyte-derived cFn conditional knockout, the number of OPCs recruited to the demyelinated lesion decreased significantly, whereas OPC numbers were unaltered following pFn conditional knockout. However, remyelination completed normally following conditional knockout of astrocyte-derived cFn and pFn. Both the EIIIA and EIIIB domains of cFn were expressed following demyelination, and *in vitro* assays demonstrated that the EIIIA domain of cFn promotes proliferation of OPCs, but not migration. Therefore, although the EIIIA domain from cFn facilitated OPC recruitment following demyelination, cFn is not essential for successful remyelination. Since previous findings indicated that astrocyte-derived Fn aggregates in chronic MS lesions inhibit remyelination, cFn removal may benefit therapeutic strategies to promote remyelination in MS.

INTRODUCTION

Multiple sclerosis (MS) is a central nervous system (CNS) disease, of which inflammation, demyelination, and axonal loss are the main pathological features. Regeneration of myelin (remyelination) involves proliferation and migration of activated oligodendrocyte progenitor cells (OPCs) to demyelinated lesions, and their subsequent differentiation into myelinating oligodendrocytes (Franklin and ffrench-Constant, 2008; Zawadzka et al., 2010). The extent of remyelination in MS is variable, but often is insufficient to prevent chronic axonal loss (Patrikios et al., 2006; Franklin et al., 2012). Promoting endogenous remyelination provides a means to reduce axonal degeneration and thereby slow progression of clinical disability (Franklin et al., 2012; Duncan et al., 2009).

Fibronectin (Fn) is an extracellular matrix (ECM) protein, that is essential for embryonic development (George et al., 1993). In healthy adults, Fn is continuously produced by hepatocytes and circulates in the plasma. Fn is absent from the healthy CNS, but expressed in demyelinated lesions. Plasma Fn (pFn) leaks across a disrupted blood-brain barrier (Sobel and Mitchell, 1989; van Horssen et al., 2005; Satoh et al., 2009), and cellular Fn (cFn) is primarily synthesized by astrocytes (Hibbits et al., 2012; Stoffels et al., 2013b), but also by microglia/macrophages and endothelial cells (Stoffels et al., 2013a). In contrast to pFn, cFn may contain alternatively spliced domains, named EIIIA, EIIIB and the V-region in rodents (EDA, EDB and IIICS, respectively, in humans) (Paul et al., 1986; Schwarzbauer et al., 1987). *In vitro,* Fn stimulates migration and proliferation of OPCs via αv integrin receptors, the only integrin receptors for Fn present on OPCs (Milner and ffrench-Constant, 1994; Milner et al., 1996; Blaschuk et al., 2000; Baron et al., 2002). The expression of both Fn and αv integrins is transiently increased in demyelinated lesions (Zhao et al., 2009). Therefore, Fn may contribute to remyelination by promoting OPC recruitment. However, removal of Fn is required for remyelination to proceed to completion, since Fn inhibits myelin sheet formation (Buttery and ffrench-Constant, 1999; Maier et al., 2005; Siskova et al., 2006; Siskova et al., 2009). In addition, Fn aggregates impair OPC differentiation *in vivo*, and likely contribute to remyelination failure in MS (Stoffels et al., 2013a).

Here, we explored the role of Fn in more depth by asking what distinctive roles the two Fn variants, pFn and astrocyte-derived cFn, play in remyelination. Using an inducible Cre-lox recombination strategy to selectively remove pFn, astrocyte-derived cFn or both (Sakai et al., 2001; Hirrlinger et al., 2006) in combination with a well-established model of CNS de- and remyelination (Blakemore and Franklin, 2008), we found that cFn from astrocytes, but not pFn, promotes recruitment of OPCs. *In vitro* analysis revealed that this was likely mediated by the EIIIA domain, which enhances OPC proliferation. However, although conditional knockout of astrocyte cFn was associated with reduced OPC recruitment following demyelination, it was not sufficient to affect remyelination outcome. The translational implication of our data therefore is that elimination of astrocyte-derived cFn may be attempted in MS to prevent Fn aggregation, which will likely be beneficial in promoting endogenous remyelination (Stoffels et al., 2013a).

MATERIAL AND METHODS

Animals

Animals were housed under standard conditions. All experiments were performed in compliance with United Kingdom Home Office regulations. Female Sprague Dawley rats at 8-10 weeks of age were obtained from Harlan, UK. Plasma Fn (pFn) inducible, conditional knockout mice (hereafter referred to as pFn^{cKO}) were a kind gift from Dr. R. Fässler, Max Planck Institute for Biochemistry, Martinsried, Germany. Inducible, conditional knockout (hereafter referred to as 'conditional knockout') of pFn was created as described (Sakai et al., 2001). Briefly, floxed Fn mice were crossed with mice expressing Cre recombinase under the control of the polyinosinic-polycitidic acid (poly I:C) responsive Mx promoter (Mx-Cre). On Cre-mediated recombination at the *loxP* sites, the start codon, signal sequence and the exon/intron border of exon 1 are removed from the Fn gene to generate the null allele (Sakai et al., 2001). Cre-mediated recombination was induced in hepatocytes from the 6-week old mice carrying Mx-Cre by two intraperitoneal injections of poly I:C (GE Healthcare, Amersham, UK) with a 48 h interval as previously described (Sakai et al., 2001). Control animals received vehicle only (PBS). Mice were subjected to lysolecithin-induced demyelination at 2-3 weeks following induction of the knockout.

Conditional knockout mice devoid of astrocyte-derived Fn (astrocyte Fn; aFn^{cKO}) were created by crossing Fn floxed mice with mice expressing Cre recombinase driven by human glial fibrilary acid protein (GFAP), with its nucleus translocation controlled by a modified estrogen receptor (hGFAP-CreERT2) (Hirrlinger et al., 2006). The hGFAP-CreERT2 mice were a kind gift from Dr. F. Kirchhoff, Max Planck Institute of Experimental Medicine, Goettingen, Germany. To induce conditional knockout of Fn from astrocytes, tamoxifen (100 mg/kg in corn oil, Sigma-Aldrich, Gillingham, UK) was injected intraperitoneally daily for 5 consecutive days, starting from 10 days prior to demyelination (Hirrlinger et al., 2006; Leone et al., 2003). The control group was injected with corn oil.

Compound astrocyte and pFn conditional knockout ($a+pFn^{cKO}$) was achieved by breeding mice heterozygous for MxCre and hGFAP-CreERT2, and homozygous for the floxed Fn gene. The induction protocol for these mice was the combination of that described for single conditional knockout strains above.

Lysolecithin-induced demyelination of the spinal cord and tissue processing

Surgery and tissue processing were performed as described (Zawadzka et al., 2010; Zhao et al., 2006). Briefly, animals at about 9-10 weeks old were anaesthetized with isoflurane, and spinal cord lesions were created by direct injection of 1 µL 1% lysolecithin into the ventral funiculus through a gap between two thoraco-lumbar vertebrae. In the conditional knockout animals, lesions were induced 1-2 weeks after completing the induction protocol. Blood samples were obtained for isolating plasma from the tail at the time of lesion, and collected in citrate-dextrose solution (Sigma-Aldrich, Dorset, UK), then stored at -80°C until use.

At the designated time post lesion, animals were euthanized by intravenous injection of

pentobarbital followed by appropriate protocols of tissue processing. For immunohistochemistry and *in situ* hybridization, animals were perfusion fixed with 4% phosphate-buffered paraformaldehyde (PFA) solution via the left ventricle, after which dissected spinal cord containing the lesions was treated with 20% sucrose in phosphate-buffered saline (PBS) overnight. Cords were embedded in OCT (Thermo Fisher Raymond Lamb, Loughborough, UK), and cut in coronal sections at 12 µm thickness. These sections were mounted on poly-L-lysine coated slides (Polysciences Europe GmbH Eppelheim, Germany) and stored at -80ºC until further use. For resin embedding and semithin sectioning, animals were perfused with 4% phosphate-buffered gluteraldehyde and subjected to a standard resin embedding process (Zhao et al., 2006). Semithin sections of 1 µm were cut and stained with alkaline toluidine blue. Ranking analysis on semithin sections of remyelinated lesions was performed by two independent, blinded researchers as described (Ibanez et al., 2004).

Glial cell cultures

Primary glial cell cultures were generated from 1-2-day old Wistar rats (Harlan, the Netherlands) as previously described (Baron et al., 2002; Bsibsi et al., 2012). Briefly, after 10-12 days in culture on poly-l-lysine (5 µg/mL, Sigma-Aldrich, St. Louis, MO) coated flasks, OPCs and astrocytes were isolated via a shake-off procedure (McCarthy and de Vellis, 1980). Contaminating microglia were removed by shaking the flasks at 150 rpm for 1 h at 37°C on an orbital shaker. Subsequently, flasks were shaken at 240 rpm overnight at 37°C. Floating OPCs were further purified by differential adhesion. Then, OPCs were cultured in defined Sato medium (Maier et al., 2005) on appropriate cell culture plastics for the different assays (see below). Purity of the OPC cultures was routinely assessed by immunocytochemistry for Olig2 (1:1000; Millipore, Billerica, MA) and cultures used were > 97% pure.

To obtain astrocytes, a subsequent overnight shake off was performed at 240 rpm and the remaining astrocyte monolayer was removed by trypsin. Astrocytes were cultured in T162 flasks (Corning Costar, Lowell, MA) in DMEM (Life Technologies, Paisley, UK) containing 10% fetal calf serum (FCS; Bodinco, Alkmaar, The Netherlands) and antibiotic supplements (Life Technologies), and passaged once before experimental use. Regular immunocytochemistry for glial fibrillary acidic protein (GFAP; Millipore; 1:500) was performed to assure sufficient purity of the astrocyte cultures (>97%).

Plasma and cellular fibronectin coatings

To obtain cFn, astrocytes were cultured on 10 cm dishes (Corning) for 48 h, followed by water-lysis at 37°C. The remaining astroglial matrix was scraped in sterile PBS containing Complete Mini protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), followed by protein concentration determination with Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard. Then, 8-well chamber-slides were incubated with 8 µg pFn from bovine serum (Sigma-Aldrich) or 8 µg astroglial matrix protein ('cFn') per well in PBS for 3 h at 37°C. Similarly, both sides of membranes on transwell microchambers (Becton-Dickinson Labware, Bedford, MA)

were coated with 10 µg of pFn or cFn per well, and 3.5 µg pFn or cFn was used per well of a 96-wells plate. When indicated, Fn coatings were incubated with functional blocking antibodies (2 µg/mL) for 1 h at 37°C, followed by a gentle wash with PBS and seeding of OPCs. Blocking antibodies used were: mouse anti-Fn IST9 (directed against the EIIIA domain, Abcam, Cambridge, UK) and mouse anti-Fn C6 (directed against the EIIIB domain, Abcam). Notably, the astrocyte-derived cFn preparation also contains other astroglial matrix proteins, such as laminin (Liesi et al., 1984; Liesi et al., 1983) and chondroitin sulphate proteoglycans (Lau et al., 2012), but although these proteins may affect OPC adhesion, proliferation and migration also (Milner et al., 1996; Lau et al., 2012; Hu et al., 2009), EIIIA and EIIIB are exclusively present in cFn.

SDS-PAGE and Western blotting

Equal amounts of plasma from pFn^{cKO} and $a+pFn^{cKO}$ mice and their controls were mixed with standard SDS sample buffer, heated for 10 min at 95°C, and separated by electrophoresis on SDS-PAGE gels (8%, Expedeon, Cambridge, UK) for 1 h at 150 V. Protein was transferred to a PVDF membrane (Invitrogen, Paisly, UK) using a wet blotting system (Expedeon, Cambridge, UK) and according glycine-Tris-methanol buffer. After three washes with 0.1% Tween-20 in PBS, membranes were blocked with 5% non-fat milk solution and incubated with a rabbit anti-Fn antibody (Millipore, Watford, UK; 1:10,000) in blocking buffer, overnight at 4°C. Membranes were washed, and incubated with HRP-conjugated anti-rabbit antibody (Roche, Lewes, UK; 1:1000) in washing buffer. Signals were detected using enhanced chemiluminescence plus (ECL plus; GE Healthcare, Amersham, UK) followed by exposure on suitable X-ray film (Thermo Scientific, Rockford, IL). For detection of mouse IgG as a control, membranes were washed and incubated with biotinylated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc, Newmarket, UK; 1:1000) for 1 h, after which the Vectastain ABC Elite kit (Vector Laboraties, Peterborough, UK) was used according to the manufacturer's instructions, and signals were developed as described above.

Immunochemistry

Frozen spinal cord sections were air dried for approximately 1 h and immunohistochemistry was performed as described (Stoffels et al., 2013a) using antibodies against Fn (Millipore; 1:1000), Olig2 (Millipore, 1:1000; R&D Systems, Abingdon, UK; 1:200), Sox2 (Santa Cruz Biotechnology Inc, Dallas, TX; 1:500), KI67 (Abcam; 1:1000), and Iba1 (Abcam; 1:500). Antigen retrieval was performed before immunostaining with Olig2, using target retrieval solution (DAKO, Ely, UK) at 95°C for 20 min. Immunochemistry on coated cFn was performed as described (Stoffels et al., 2013a), using antibodies against Fn (1:50), IST9 (1:50) and C6 (1:50). Secondary antibodies used were appropriate Alexa Fluor© (488 or 594)-conjugated secondary antibodies (Invitrogen; 1:500).

For immunohistochemistry using primary antibodies generated in mice on mouse tissue, a modified protocol was used. This protocol was applied for primary antibodies against Nkx2.2 (Hybridoma Bank Iowa, Iowa City, IA; 1:100), CC1 (Millipore; 1:100) and Aldh1L1 (NeuroMab, Davis,

CA; 1:100) and secondary antibodies used were Alexa Fluor© 488-conjugated-anti IgG2b (for Nkx2.2 and CC1) or -anti IgG1 (for Aldh1L1) (Invitrogen; 1:400). Sections were first permeabilized with 1% Triton-X-100 in 0.05 M Tris-buffered saline (TBS) for 30 min. Then, sections were blocked for 30 min in TBS with 10% normal goat serum (NGS) and 0.25% Triton-X-100, followed by another blocking step for 1 h using mouse-on-mouse Ig solution (Vector Laboratories) according to the manufacturer's instructions. Primary antibodies were appropriately diluted in TBS containing 2% NGS and 0.3% Triton-X-100, and applied for 1 h. Secondary antibodies were appropriately diluted in TBS with 1% NGS, 0.1% Triton-X-100 and DAPI (1 µg/mL), and applied for 15 min, followed by a 10 min incubation in 0.1% Sudan Black in 70% ethanol. All incubations were at room temperature. After each step, sections were washed in 0.05 M TBS with 0.1% Tween-20 (VWR, Lutterworth, UK) 3 times 2 min.

Images from immunohistochemistry with either protocol were acquired using a Zeiss Observer A1 fluorescent microscope, and images from immunochemistry on coatings were acquired using a Zeiss Axioskop 2 microscope with Leica Application Suite V3 software. For counting cell numbers, in each animal 3 demyelinated lesions were selected, spanning the centre of lesions, at approximately 120 µm distance from each other. The outline of each lesion was defined based on the increase in DAPI cellularity inside the lesion using Zeiss Axovision 4.8 software (Stoffels et al., 2013a). The numbers of marker-positive cells inside the lesions were manually counted 3 times and averaged for each lesion. There were 4-5 animals per group. Individual Fn- and Iba1-immunereactive cells could not be discerned reliably, hence these were quantified by measuring the optical densities from immunofluorescence using FIJI software.

Reverse transcription-PCR

Total RNA was isolated from tissue homogenates using the RNeasy Mini kit (Qiagen). Reverse transcription of 0.5 µg total RNA was performed in the presence of oligo(dT)12–18 and dNTPs (Invitrogen) with SuperScript_II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting complementary DNA was amplified using Microzone MegaMix Blue (Microzone Ltd, West Sussex, UK) and the following primers: for EIIIA; forward: 5'-AAACAGAAATGACCATTGAAGTTTG-3', reverse: 5'-TTGATTTCTTTCATTGGTCCTGTCTT-3'; for EIIIB; forward:5'-TTACACTGTCAAAGATGACAAGGAAA-3',reverse:5'-TGACATCAGAAGAATCAAAACCAGTT-3' (optimized from: (Magnuson et al., 1991; Vitale et al., 1994), and for cyclophilin; forward: CTCGTGACCCCTCTTTCC, reverse: CATTATTTTCCTCATTTCCCCT (Stoffels et al., 2013a; Zhao et al., 2006). Cycling conditions were optimized, with all cycle numbers falling within the linear production range, and PCR products were resolved by agarose gel electrophoresis. Changes in gene expression were analyzed by Scion Image Software. The EIIIA and EIIIB primers generate two products: a band that includes the alternatively spliced domain (526 bp for EIIIA, 640 bp for EIIIB) and another band when these domains are spliced out (256 bp for EIIIA, 367 bp for EIIIB) (Vitale et al., 1994). Therefore, the upper bands were used for quantifications. Hereto, optical densities were normalized against the corresponding value for the housekeeping gene cyclophilin obtained in separate reactions.

BrdU incorporation assay

OPCs were plated on 8-well Permanox chamber-slides (Nunc, Naperville, IL), pre-coated with 5 µg/mL poly-l-lysine followed by appropriate cFn or pFn coatings (described above), at a density of 30,000 cells per well. OPCs were allowed to incorporate 5-bromo-2-deoxyuridine (BrdU; 10 µM; Roche) for 24 h in the presence of 10 ng/mL PDGF-AA and 10 ng/mL FGF-2. Then, cells were fixed in 4% PFA for 20 min, and additionally fixed in 5% acetic acid in ethanol for 20 min. BrdU was detected using reagents from the BrdU Labelling and Detection Kit I (Roche) according to the manufacturer's instructions with the addition of the oligodendrocyte lineage marker Olig2 (Abcam) and Alexa Fluor© 546-conjugated anti-rabbit antibody, and visualization of nuclei with DAPI (1 µg/mL). To compare the percentages of BrdU-incorporating cells between the conditions, the numbers of double BrdU- and Olig2-positive cells were counted relative to the Olig2-positive cells (at least 150 cells per condition) from images captured with a Leica TCS SP8 Confocal Laser Scanning Microscope.

Transwell migration assay

OPCs were seeded on polyethylene terephthalate membranes of 8 µm pore size (Becton-Dickinson Labware) in 12-well modified Boyden transwell microchambers at a density of 80,000 cells per insert. OPCs were allowed to migrate through the coated (described above) membranes for 4 h using 10 ng/mL PDGF-AA as a chemoattractant in the bottom of the well. Non-migrating cells were removed from the top compartment with a cotton swab. Remaining cells in the membranes were fixed for 20 min in 5% acetic acid in ethanol and nuclei were visualized using DAPI (1 µg/mL) in PBS for 30 min. After washing in PBS, membranes were mounted on glass slides and images of DAPI-positive, migrated cells were captured using a Zeiss Axioskop 2 plus microscope with Leica Application Suite v3 software (15 X20 fields per membrane). The numbers of cells were assessed using FIJI software.

Adhesion assay

Coated 96-wells plates (Nunc) were blocked for 30 min with 1% heat-inactivated BSA at 37°C. Then, wells were left untreated or treated with the Fn blocking antibodies against EIIIA or EIIIB (mouse anti-Fn IST9; Abcam and anti-Fn C6; Abcam). After washing, 10,000 OPCs in 50 µl Sato medium per well were allowed to adhere for 1 h at 37°C. For integrin blocking experiments, OPCs were pre-incubated with anti-integrin β1 (Becton Dickinson Pharmingen, Breda, NL; 1:200), anti-integrin β3 (Becton Dickinson Pharmingen; 1:200) or anti-integrin β5 (Millipore, Chemicon, Temecula, CA; 1:200) antibodies for 30 min at 37°C. The cells were washed two times with PBS, and adhered cells were fixed for 15 min with ice-cold methanol. Cells were stained with 0.2% crystal violet (in 2% ethanol; Sigma), then washed several times with water, after which the stain was solubilised in 1% SDS. Adhesion was quantified by measuring the absorbance at 570 nm after 30 min. Adhesion is expressed as percentage of the corresponding untreated substrate control resulting from triplicate determinations.

In situ hybridization

The DIG-labeled PLP probe was generated as described (Chari et al., 2006). Briefly, mouse PLP cDNA (a kind gift from I.R. Griffiths, Glasgow, UK) was used, subcloned into the pGEM4 plasmid (Promega, Southampton, UK). The probe was labelled using a DIG RNA labelling kit (Roche, Lewes, UK). *In situ* hybridization was performed as described previously (Sim et al., 2000; Zhao et al., 2008). Methods of quantification for the *in situ* hybridized sections are described under 'immunochemistry', except that measurements of the outlines of the lesions were based on the darker, lesioned tissue background.

Statistical analyses

Statistical analyses were performed in GraphPad Prism (GraphPad Inc, La Jolle, CA). First, the Kolmogorov-Smirnov test was applied to test for a normal distribution of the data. Multiple group comparisons of data, which could thus be assumed to have a normal distribution (data sets of Fn^{cKO} animals, derived from immunohistochemistry and *in situ* hybridization studies, except for the data describing immunofluorescence intensity of Fn- and Iba1-staining), were performed using one-way ANOVA followed by Tukey's Multiple Comparison Test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Multiple group comparisons of data that were not compatible with a normal distribution (data sets derived from *in vitro* assays and reverse transcription PCR on demyelinated tissue, as well as of the data describing immunofluorescence intensity of Fn and Iba1 stainings) were performed using the Kruskal-Wallis test followed by Dunn's Multiple Comparison Test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Non-parametric ranking data, derived from blindly ranking alkaline toluidine blue stained resin sections for estimated degrees of remyelination, were statistically analysed using the Mann-Whitney test (Ibanez et al., 2004). For the *in vivo* rodent data from immunohistochemistry, *in situ* hybridization and reverse transcription PCR studies, a representative graph of 2-3 independent experiments is shown, displaying absolute means of 3-4 animals per group and 3 lesions per animal, each approximately 120 µm from each other, with the exception of immunochemistry for Iba1, data of which are shown as a relative to the mean immunofluorescence levels of littermate controls, set at 100% for each independent experiment. Quantification methods are described under 'immunochemistry'. For *in vitro* proliferation, migration and adhesion assays, graphs display mean values relative to a control condition (cFn or pFn) from 3-5 independent experiments. All error bars represent standard deviations.

RESULTS

Fibronectin in demyelinated lesions is predominantly produced by astrocytes

Transient Fn expression during demyelination is hypothesized to benefit remyelination (Hibbits et al., 2012; Stoffels et al., 2013a; Zhao et al., 2009), whereas the persistent presence of Fn aggregates, inhibit OPC differentiation and impairs remyelination (Stoffels et al., 2013a). The aim of the present study was therefore to better define the potential involvement and underlying mechanism(s) of the different Fn sources in remyelination. We used a well-established rodent model of experimental

demyelination, in which focal, primary demyelination is created by the injection of lysolecithin into the white matter of the spinal cord ventral funiculus (Zawadzka et al., 2010; Blakemore and Franklin, 2008). Spontaneous and complete remyelination of these lesions proceeds over a period of approximately 21-28 days in young adult rodents, which involves proliferation and migration of local, activated OPCs to the demyelinated lesions ('recruitment'; 1-10 days post lesion (DPL)) (Franklin and ffrench-Constant, 2008; Zawadzka et al., 2010), followed by differentiation of oligodendrocytes and myelin sheath formation (10-21 DPL) (Zhao et al., 2006). In order to eliminate pFn and cFn from lysolecithin-demyelinated lesions, we used Cre/lox technology, creating conditional knockout adult mice devoid of plasma Fn (pFn^{cKO}), astrocyte Fn (aFn^{cKO}) or both ($a+pFn^{cKO}$). The pFn null allele was generated by activation of the Mx-Cre promoter with poly I:C as described (Sakai et al., 2001), which removes the start codon, signal sequence and the exon/intron border of exon 1 from the pFn gene. In order to remove cFn, we targeted astrocytes, because these are considered a major source of Fn in the CNS following injury, and synthesize pathological Fn aggregates in MS (Hibbits et al., 2012; Stoffels et al., 2013a). We thus used mice expressing Cre recombinase under control of a GFAP promoter, which was rendered active by exposure to tamoxifen via a modified estrogen receptor. The induction efficiency was tested in a reporter line, as described in a previous report (Hirrlinger et al., 2006). In our own experiments, this approach showed that at 5 days after a course of tamoxifen administration in unlesioned adult mice, of astrocytes in spinal cord white matter on average 78.5% ± 2.4% SD (n=4) expressed the reporter (yellow fluorescent protein), indicating an efficient recombination.

Using this approach, pFn was successfully eliminated from plasma in pFn^{cKO} mice, as revealed by Western blot analysis (Fig. 1A). Similar results were obtained on plasma from $a+pFn^{cKO}$ mice, whereas pFn remained present in the plasma of aFn^{cKO} mice as expected (Fig. 1B). Given the virtual absence of cFn from the healthy CNS and its increased expression on demyelination (Stoffels et al., 2013a; Zhao et al., 2009), we assessed local Fn expression in the CNS of aFn^{cKO} and $a+pFn^{cKO}$ mice by immunohistochemistry of lysolecithin-induced demyelinated lesions. As shown in Fig. 1C and 1D, Fn expression was increased in demyelinated lesions of control animals at 5 DPL as reported in rat models (Stoffels et al., 2013a; Zhao et al., 2009). In pFn^{cKO} mice, Fn immunostaining was unaltered compared to expression in littermate controls (Fig. 1C,D), despite the strong reduction in Fn plasma levels (Fig. 1A). These data indicate that either pFn is not a major source of Fn expression within lesions, or that cFn may compensate for the absence of pFn. In contrast, Fn expression was markedly reduced in demyelinated lesions of aFn^{cKO} and $a+pFn^{cKO}$ mice (Fig. *1C*,D), corroborating astrocytes as a major source of cFn after demyelination. Having reduced Fn levels from plasma and astrocytes, we next analyzed how remyelination was affected at both the recruitment and differentiation stages of remyelination.

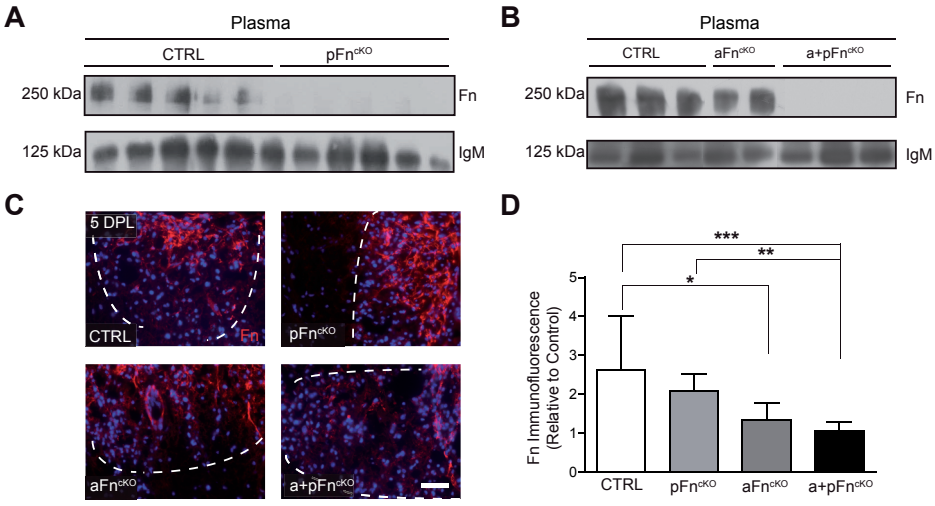
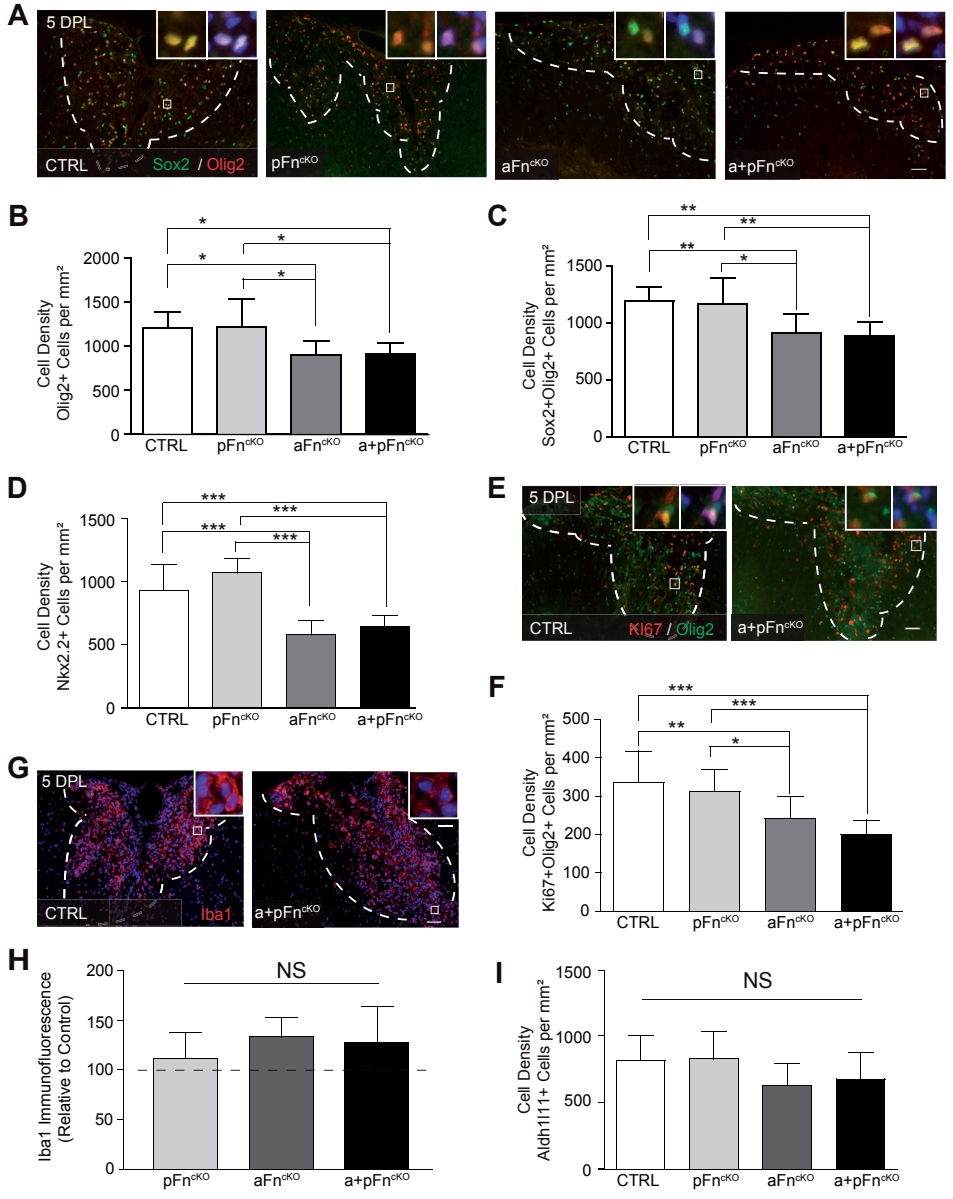


Figure 1. Fibronectin in demyelinated lesions is predominantly produced by astrocytes. A,B. Western blot analysis for fibronectin (Fn) and immunoglobulin M (IgM) on plasma of littermate controls (CTRL) and after conditional knockout of Fn from plasma (pFn^{cKO}), astrocytes (aFn^{cKO}) or astrocytes and plasma (a+pFn^{cKO}). Note the virtual absence of Fn from plasma after pFn^{cKO} and a+pFn^{cKO}. C. Immunohistochemistry for Fn on lysolecithin-demyelinated spinal cord lesions at 5 days post lesion (DPL) after pFn^{cKO}, aFn^{cKO}, a+pFn^{cKO} or in CTRL mice. D. Quantification of immunofluorescent staining intensities in (C) from 3 animals per group, and 3 lesions per animal, approximately 120 µm distant from each other from images that were taken on the same day using the exact same camera settings. Note the reduction in intensity of immunofluorescence relative to CTRL after aFn^{cKO} and a+pFn^{cKO}, but not following pFn^{cKO}. Bars represent means. Error bars show standard deviations. Statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's Multiple Comparison Test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Demyelinated lesion outlines were measured in Zeiss Axovision 4.8 software based on the increase in DAPI staining inside the lesions, and outlines are represented by dashed white lines. Scale bar is 50 µm.

Proliferation of oligodendrocyte progenitor cells in response to demyelination is decreased in astrocyte fibronectin conditional knockout animals

To determine whether recruitment of OPCs to the demyelinated area is affected by pFn^{cKO} and/or aFn^{cKO}, we analyzed OPC numbers after lysolecithin-induced demyelination in the different Fn^{cKO} mice. Immunostaining for Olig2 was used as a marker for oligodendrocyte lineage cells (Arnett et al., 2004; Fancy et al., 2009) and Sox2 and Olig2 double immunohistochemistry to identify OPCs (Kondo and Raff, 2004; Shen et al., 2008) (Fig 2A). This approach revealed a small, significant reduction in Olig2-positive (Olig2+) cells and Sox2+Olig2+ cells after aFn^{cKO}, but not pFn^{cKO} at 5 DPL (Fig. 2A-C). A similar reduction in Olig2+ and Sox2+Olig2+ cells was detected after a+pFn^{cKO} (Fig. 2A-C), indicating that the decrease in OPC numbers was associated with the decrease in astrocyte cFn. An additional decrease in pFn levels in a+pFn^{cKO} did not further reduce the number of OPCs. In support of these observations, by determining OPC numbers using the transcription factor Nkx2.2 as a marker (Watanabe et al., 2004), we similarly detected that OPC numbers were reduced after aFn^{cKO} and



a+pFn^{cKO} (Fig. 2D). The lesions were of comparable sizes in the different Fn^{cKO} samples relative to controls (data not shown). In tissue culture, pFn promotes OPC migration and proliferation (Milner et al., 1996; Baron et al., 2002; Hu et al., 2009). To examine whether the reduction in OPC numbers in aFn^{cKO} animals reflected an impairment in proliferation, we next analyzed the numbers of proliferating OPCs at 5 DPL, determined by co-labelling with Olig2 and the cell proliferation marker KI67 (Gerdes et al., 1983). This revealed a significant reduction in KI67+Olig2+ cells in aFn^{cKO} and a+pFn^{cKO}, but not pFn^{cKO} (Fig. 2E,F), indicating that the decrease in OPC numbers is, at least partly, a

Figure 2. Proliferation of oligodendrocyte progenitor cells is reduced after conditional knockout of astrocyte fibronectin, but not after plasma fibronectin conditional knockout alone. A-I. After 5-days post lysolecithin-induced demyelination (5 DPL) of the mouse spinal cord ventral funiculus, the density of Olig2-positive ('Olig2+', B), Sox2+Olig2+ (A,C), Nkx2.2+ (D), KI67+Olig2+ (E,F) Iba1+ (G,H), and Aldh1L1+ (I) cells were determined by immunohistochemistry. Note the decrease in Olig2+, Sox2+Olig2+, Nkx2.2+ and KI67+Olig2+ cells number after conditional knockout of fibronectin from astrocytes (aFn^{cKO}) or from astrocytes and plasma ($a+pFn^{cKO}$), but not from plasma alone (pFn^{cKO}) relative to littermate controls (CTRL). Images in A,E and G are representative images of demyelinated areas in the different groups. Insets show higher power magnifications of the double positive cells that were counted, with the blue color representing DAPI staining. The outline of the demyelinated lesions was measured in Zeiss Axovision 4.8 software based on increased DAPI stainings inside lesions and outlines are represented by dashed white lines. Double (A,C,E,F) or single (B,D,G-I) positive cell numbers were manually counted 3 times for 4 animals per group, and 3 lesions per animal, approximately 120 µm distant from each other. Representative graphs of 2-3 independent experiments are shown. Bars represent means. Error bars show standard deviations. Statistical analyses were performed using one-way ANOVA, followed by Tukey's Multiple Comparison Test (* $p < 0.05$; ** $p<0.01$; *** $p < 0.001$; NS: not significant). Scale bars are 50 µm.

result of impaired proliferation. In contrast, we did not observe a substantial change in microglia/macrophage numbers after Fn^{cKO}, as assessed by immunohistochemistry for ionized calcium-binding adaptor molecule 1 (Iba1) (Imai et al., 1996) (Fig. 2G,H). Similarly, astrocyte numbers were not significantly affected by Fn^{cKO}, as reflected by comparable intense immunofluorescence levels of aldehyde dehydrogenase 1, member L1 (Aldh1L1) (Lovatt et al., 2007; Cahoy et al., 2008) (Fig. 2I). Hence, aFn^{cKO} resulted in a reduced number of OPCs after demyelination, whereas microglia/macrophage and astrocyte cell numbers were unaffected.

Oligodendrocyte differentiation and remyelination are not affected by conditional knockout of fibronectin from astrocytes

We next examined whether the decreased OPC numbers observed in aFn^{cKO} and $a+pFn^{cKO}$ during demyelination resulted in altered CNS remyelination following lysolecithin-induced demyelination. Oligodendrocyte lineage cell numbers were analyzed by immunohistochemistry for Olig2 at 14 days post lysolecithin-induced demyelination (Zhao et al., 2006). As shown in Fig. 3A and 3B, Olig2+ cells were still significantly decreased in $a+pFn^{cKO}$ and aFn^{cKO} as compared to littermate controls. In pFn^{cKO} animals, Olig2+ cell numbers did not differ from the control animals in the lesions. Furthermore, analysis of differentiated oligodendrocytes, determined by immunohistochemical staining for CC1 (Bhat et al., 1996), showed that the numbers of CC1+ cells were identical in all groups (Fig. 3C). This was confirmed using an alternative marker of mature oligodendrocytes, *in situ* hybridization for proteolipid protein (PLP) mRNA, a major myelin protein, which revealed no differences in PLP mRNA+ cells (Fig. 3D,E). Therefore, despite a reduction of OPCs after demyelination in aFn^{cKO} and $a+pFn^{cKO}$, this was not sufficient to result in an impairment of oligodendrocyte generation at 14 DPL.

To assess whether myelin sheath formation was affected, we examined toluidine blue stained semithin resin sections of lesions from control and $a+pFn^{cKO}$ animals at 21 DPL. We could not detect

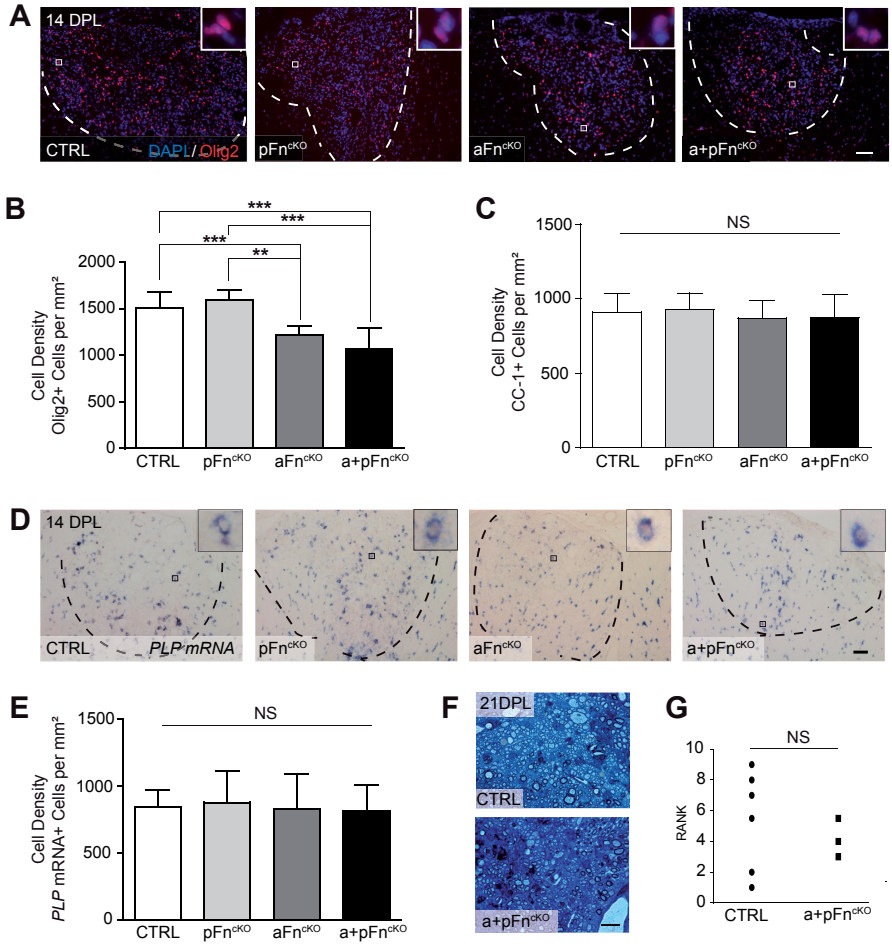


Figure 3. Oligodendrocyte differentiation and remyelination are not affected by conditional knockout of astrocyte fibronectin. A-E. At 14 days post lysolecithin-induced demyelination (14 DPL) of the spinal cord ventral funiculus, immunohistochemistry (A-C) and *in situ* hybridization (D,E) were applied to determine the numbers of Olig2+ (A,B), CC1+ (C) and *PLP* mRNA+ (D,E) cells after conditional knockout of fibronectin from plasma (pFn^{cKO}), astrocytes (aFn^{cKO}), or astrocytes and plasma (a+pFn^{cKO}). Note the significant decrease of Olig2+ oligodendrocyte lineage cells after aFn^{cKO} and a+pFn^{cKO} (A,B), whereas the numbers of differentiating oligodendrocytes dot not differ from littermate controls (CTRL) (C-E). Images in A and D are representative images of demyelinated areas in the different groups. Insets show higher power magnifications of the positive cells that were counted, with the blue color representing DAPI staining. Outlines of demyelinated lesions were measured in Zeiss Axovision 4.8 software based on the increase in DAPI staining inside lesions, and outlines are represented by dashed lines. Cell numbers were manually counted 3 times from 4 animals per group, and 3 lesions per animal, approximately 120 µm distant from each other. Representative graphs of 2-3 independent experiments are shown. Bars represent means. Error bars show standard deviations. Statistical analyses were performed using one-way ANOVA, followed by Tukey's Multiple Comparison Test (** < 0.01; *** < 0.001; NS: not significant). Scale bars are 50 µm. F,G. At 21 DPL, semi-thin sections of resin-embedded CTRL and a+pFn^{cKO} animals were stained with alkalin toluidin blue to analyze the myelin structure (F), and blindly ranked according to estimated percentage of remyelination by two independent researchers, after which statistical differences were analyzed using the Mann Whitney test (G). Scale bar is 100 µm. NS means 'not significant'

any gross morphological differences between remyelinated lesions from control and a+pFn^{cKO} animals (Fig. 3F). Ranking analysis of the degree of remyelination (Ibanez et al., 2004) did not reveal significant differences between controls and a+pFn^{cKO} lesions either (Fig. 3G). Therefore, cFn from astrocytes, although contributing to OPC proliferation, is not essential for remyelination.

Expression of the alternatively spliced domains EIIIA and EIIIB mRNA of cellular fibronectin is increased following demyelination

We next investigated the specific contribution of the alternatively spliced domains EIIIA and EIIIB, exclusively localized within the cFn structure (Paul et al., 1986; Schwarzbauer et al., 1987) in the regulation of OPC proliferation following demyelination. We first determined the expression of both domains following demyelination by semi-quantitative analysis of mRNA expression. Using primers against exons flanking the alternatively spliced domains, two bands were detected: a long product containing the alternatively spliced domain (used for quantification) and a short product lacking the alternatively spliced domain (Magnuson et al., 1991; Vitale et al., 1994) (Fig. 4A). Relative to unlesioned tissue, EIIIA and EIIIB mRNA levels were increased in demyelinated tissue at 5 DPL (Fig. 4A-C), similar to our previous report for total Fn protein and Fn EIIIA protein (Stoffels et al., 2013a). Furthermore, during remyelination (14 DPL), EIIIB mRNA levels returned to the levels observed in

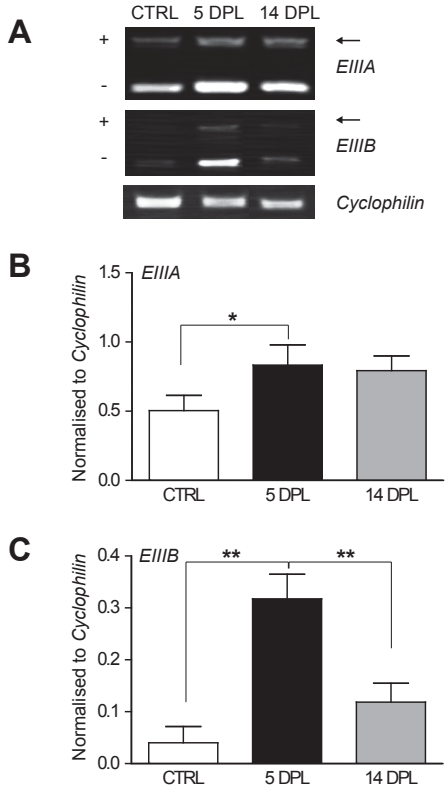


Figure 4. Enhanced expression of fibronectin alternatively spliced domains EIIIA and EIIIB mRNA after lysolecithin-induced demyelination. A. Reverse transcription PCR on spinal cord RNA from control (CTRL) rats or at 5 days (5 DPL) or 14 days (14 DPL) post lysolecithin -induced demyelination, showing an increase in fibronectin *EIIIA* and *EIIIB* mRNA at 5 DPL (upper bands). Note that, whereas fibronectin *EIIIB* mRNA decreases at ongoing remyelination (14 DPL), upregulation of fibronectin *EIIIA* mRNA is sustained. B,C. Quantifications of A for *EIIIA* (B) and *EIIIB* (C) fibronectin mRNA. Relative optical densities were normalized against the housekeeping gene *cyclophilin* (A) for 3-4 animals per condition. Representative graph of a duplicate experiment is shown. Statistical analysis was performed using the Kruskal-Wallis Test, followed by Dunn's Multiple Comparison Test (* $p < 0.05$; ** < 0.01).

unlesioned tissue (Fig. 4A,C; 14 DPL), whereas EIIIA levels remained increased for longer (Fig. 4A,B; 14 DPL). The high EIIIA mRNA levels at 14 DPL contrasted with the reduced levels of EIIIA protein as compared to 5 DPL that we previously reported (Stoffels et al., 2013a), implying translational regulation. Because both EIIIA and EIIIB mRNA levels are increased at 5 DPL, when OPCs are recruited, and since these elements are exclusively present in cFn, we next examined the extent to which cFn EIIIA, EIIIB or both are involved in proliferation and/or migration of OPCs.

The EIIIA domain of astrocyte-derived fibronectin promotes proliferation of oligodendrocyte progenitor cells, but not their migration and adhesion.

To examine the effect of pFn and cFn on OPC proliferation, we used commercially available pFn from bovine plasma and cFn derived from extracellular deposits of primary astrocytes (Fig. 5A, 'Fn'). The astrocyte-derived cFn preparation contained the alternatively spliced domain EIIIA (Fig. 5A, 'EIIIA') and EIIIB (Fig. 5A, 'EIIIB'), as confirmed by immunostaining with domain-specific antibodies. Functional blocking antibodies against EIIIA ('IST9', Liao et al., 1999) and EIIIB ('C6', Balza et al., 2009) were used to eliminate signals from these domains. OPCs were allowed to proliferate on the different substrates in the presence of PDGF-AA and FGF-2 for 24 h, after which percentages of BrdU-incorporating Olig2+ cells were determined by immunochemistry as a measure of OPC proliferation. OPCs proliferated equally well on either cFn (16% ± 5% SD) or pFn (15% ± 8% SD) (Fig. 5B). However, proliferation of OPCs was markedly reduced when the cells were cultured on cFn with the EIIIA domain blocked (Fig. 5B; 'cFn+IST9'). In contrast, addition of the EIIIA blocking antibody to pFn did not affect OPC proliferation (Fig. 5B; 'pFn+IST9'), indicating that the reduction in OPC proliferation following the blocking of EIIIA with the IST9 antibody was attributable to the specific effect(s) of the antibody. Moreover, blocking EIIIB from cFn (Fig. 5A; 'EIIIB') with the C6 antibody (Fig. 5B; 'cFn+C6') did not affect OPC proliferation. To determine whether EIIIA and/or EIIIB of cFn are also important for OPC migration, we allowed OPCs to migrate through transwell microchambers, containing membranes coated with pFn or cFn on both sides, using PDGF-AA as an attractant in the bottom well. As shown in Fig. 5C, migration of OPCs was similar on cFn and pFn, and was not affected by blocking the EIIIA or EIIIB domains. These experiments demonstrate that EIIIA, but not EIIIB, is primarily required for proliferation of OPCs and that neither domain is essential for migration of OPCs on astrocyte-derived cFn.

Proliferation of OPCs on pFn is largely mediated by the integrin receptors αvβ3 and αvβ1 (Milner et al., 1996; Blaschuk et al., 2000; Baron et al., 2002). Although EIIIA does not directly bind to αvβ1 or αvβ3, it has been reported that EIIIA may indirectly promote adhesion of cFn to these integrins, thereby facilitating proliferation (Manabe et al., 1999). To test this possibility, we assessed whether adhesion of OPCs to cFn indirectly involved integrin β binding by using blocking antibodies against integrins β1, β3 and β5, and whether such adhesion was facilitated by functional EIIIA and EIIIB domains. Adhesion of OPCs to cFn was only modestly reduced when the integrins β3 (Fig. 5D; 'cFn+ β3') and β5 (Fig. 5D; 'cFn+ β5') were blocked, when compared to adhesion to pFn (Fig. 5D; 'pFn+

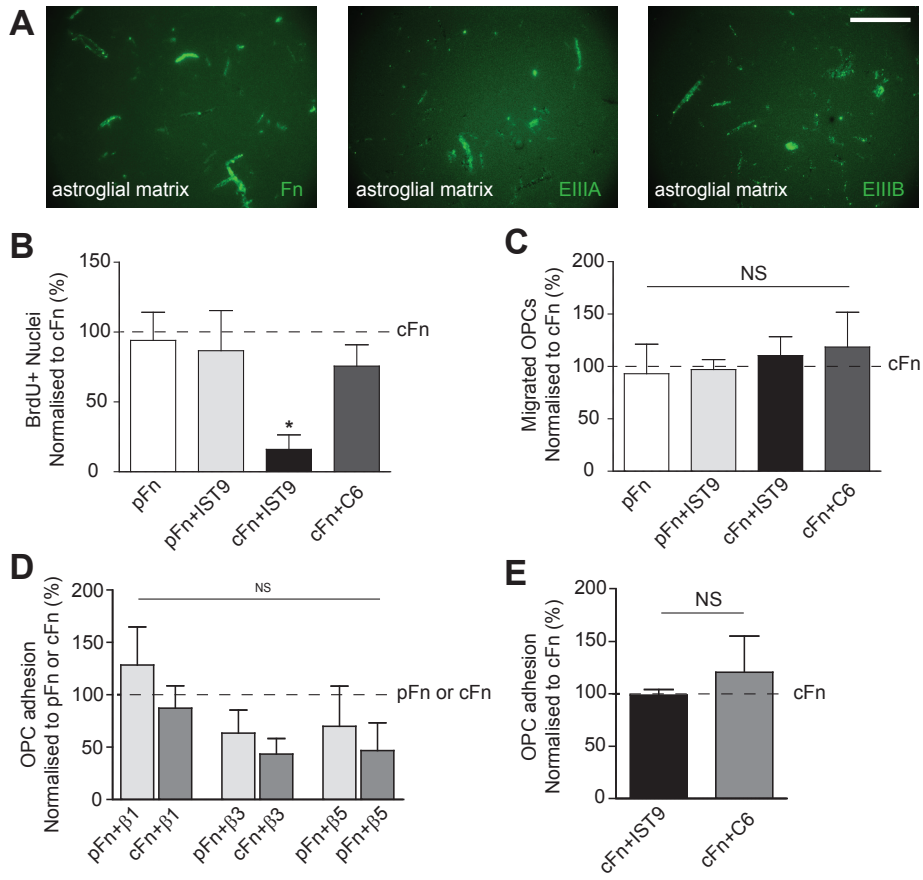


Figure 5. The EIIIA domain of astrocyte-derived fibronectin promotes proliferation of oligodendrocyte progenitor cells, but not their migration and adhesion. A. Astroglial matrix derived from neonatal rat astrocytes (cFn) immunostained for total fibronectin ('Fn'), and the alternatively spliced EIIIA ('EIIIA') or EIIIB ('EIIIB') domains. Scale bar is 50 μM. B. Oligodendrocyte progenitor cells (OPCs) from neonatal rats were cultured for 24 h on plasma fibronectin (pFn) or cFn, alone or in the presence of blocking antibodies against the EIIIA or EIIIB domains and subjected to a BrdU incorporation assay combined with Olig2 immuncytochemistry. On cFn, 16% of OPCs proliferated on average ± 5% SD. Note that addition of an EIIIA blocking antibody ('cFn+IST9') decreased the number of Olig2+ OPCs incorporating BrdU on cFn, but not on pFn ('pFn+IST9'), and BrdU incorporation did not differ between the other conditions. C. OPCs were allowed to migrate through transwell inserts coated with cFn or pFn for 4 h, alone or in the presence of blocking antibodies against EIIIA ('pFn+IST9'; 'cFn+IST9') or EIIIB ('cFn+C6') antibodies. Note that the numbers of migrated cells did not differ between the conditions. D,E. OPCs were allowed to adhere to pFn or cFn in the presence of blocking antibodies against integrins β1 ('pFn+ β1','cFn+ β1'), β3 ('pFn+ β3','cFn+ β3') or β5 ('pFn+ β5','cFn+ β5') (D) or after blocking the EIIIA ('cFn+IST9') or EIIIB ('cFn+C6') (E) domains of cFn. Note that whereas integrins β3 and β5 likely mediate adhesion of OPCs, particularly to cFn (D), adhesion does not require a functional EIIIA or EIIIB domain on cFn (E). Bars in the graphs represent means of 3-4 independent experiments relative to cFn, which was set at 100% (dashed line) or pFn for the OPCs grown on pFn in figure 5D. Error bars show standard deviations. Statistical analyses were performed using the Kruskal-Wallis Test, followed by Dunn's Multiple Comparison Test (** $p < 0.01$; NS: not significant).

β3', 'pFn+ β5'). In contrast, integrin β1 had no effect on adhesion of OPCs to both pFn and cFn (Fig. 5D; 'pFn+ β1', 'cFn+ β1'). Furthermore, functional blocking of EIIIA and EIIIB domains did not alter adhesion of OPCs to cFn (Fig. 5E). Thus, whereas the integrin β3 and β5 receptors likely mediate adhesion of OPCs to cFn, adhesion of OPCs does not require functional EIIIA or EIIIB domains of cFn.

DISCUSSION

In this study, we investigated how Fn, derived from plasma (pFn) or cellular fibronectin (cFn) derived from astrocytes, modulates remyelination following CNS demyelination. After conditional knockout of Fn from plasma (pFn^{cKO}), astrocytes (aFn^{cKO}) or both ($a+pFn^{cKO}$), astrocyte-derived cFn, but not pFn, promotes recruitment of OPCs following demyelination. Our *in vitro* analyses of OPCs, cultured on either pFn or astrocyte-derived cFn, revealed that the alternatively spliced EIIIA domain, exclusively present in cFn, is instrumental in stimulating proliferation. Our data further showed that this control of proliferation by the EIIIA domain was likely not related to an ability of EIIIA to enhance adhesion of OPCs, although integrins β3 and β5 mediate adhesion of OPCs to cFn. Furthermore, we did not observe an effect of cFn on migration of OPCs. Despite a reduction in oligodendrocyte lineage cells on $a+pFn^{cKO}$ at later stages of the remyelination process, both pFn and astrocyte cFn were not required for oligodendrocyte differentiation and complete remyelination.

Although both EIIIA and EIIIB are largely absent from healthy tissue after embryonic development, reappearance of cFn with the alternatively spliced EIIIA and EIIIB domains occurs after injury of different tissue types (ffrench-Constant et al., 1989; Jarnagin et al., 1994; Nickeleit et al., 1996; Ulrich et al., 1997). Injury-induced expression of cFn containing EIIIA is associated with increased cell proliferation, as demonstrated in a variety of cell types (Manabe et al., 1999; Stenzel et al., 2011; Olsen et al., 2012). EIIIA could theoretically facilitate proliferation of OPCs on cFn via binding to the integrins α9β1 (Olsen et al., 2012; Ou et al., 2013; Sun et al., 2013) and α4β1 (Liao et al., 2002), or by enhancing the binding affinity of other integrin receptors for Fn, most notably α5β1 and αvβ3 (Manabe et al., 1999; Xia and Culp, 1995; Manabe et al., 1997). However, integrins α4β1 and α9β1 are not expressed by OPCs or oligodendrocytes (Milner and ffrench-Constant, 1994) and the EIIIA domain was not important for adhesion of OPCs to cFn, implying that EIIIA mediates proliferation of OPCs through an alternative mechanism. This mechanism may involve other domains of Fn, since alternative splicing of Fn can change the conformation of Fn, affecting the presentation of binding sequences and exposing cryptic binding sites (Pickford and Campbell, 2004; Ventura et al., 2010). In addition to EIIIA, expression of the EIIIB domain was also upregulated after demyelination. Whereas EIIIB appears not to have a role in proliferation and migration of OPCs, its functional involvement in processes other than proliferation and migration cannot be excluded and merits further investigation.

The apparent redundancy of pFn for recruitment of OPCs observed in our *in vivo* studies was unexpected, given that stimulation of cell proliferation and migration by pFn is well documented for several cell types (To and Midwood, 2011; von Au et al., 2013), including OPCs (Milner et al.,

1996; Baron et al., 2002; Hu et al., 2009). Our *in vitro* proliferation studies also suggested that pFn and cFn are equally capable of promoting OPC proliferation. After pFn^{cKO}, a compensatory increase in cFn may have occurred, mediated by astrocytes, microglia/macrophages and endothelial cells (Stoffels et al., 2013a). From these cell types, astrocytes are thought to represent the major source of Fn after toxin-induced demyelination (Hibbits et al., 2012; Stoffels et al., 2013a), which is further supported in this study, given the pronounced reduction in Fn levels after aFn^{cKO}. Therefore, a compensatory increase in cFn could explain the absence of a clear phenotype after pFn^{cKO}, in agreement with studies of other tissues (Sakai et al., 2001). However, the observation that $a+pFn^{cKO}$ does not substantially amplify the reduction in OPC numbers during demyelination may support an alternative explanation, namely that excessive leakage of pFn to the demyelinated area does not occur on lysolecithin-induced demyelination, despite breakdown of the blood-brain barrier (Ford et al., 1990). Hence, in contrast to previous studies, in which pFn was considered a predominant source of the Fn matrix in tissue (Moretti et al., 2007), our findings indicate that cFn rather than pFn is the major component of the Fn matrix expressed after toxin-induced CNS demyelination. In MS lesions, where immune-mediated blood-brain barrier disruption is more diffuse, pFn may be a more prominent source of Fn.

Although the transient expression of cFn from astrocytes promotes recruitment of OPCs after remyelination, cFn is not required for remyelination. Relative redundancy of astrocyte-derived cFn, in spite of decreased OPC proliferation after aFn^{cKO}, is consistent with the concept that OPCs are normally recruited in excess relative to the numbers required for remyelination following small, focal, toxin-induced lesions (Franklin and ffrench-Constant, 2008; Stidworthy et al., 2004). In addition, functional compensation may result from the increased expression of several other ECM proteins in demyelinated lesions (Zhao et al., 2009). In support of this, osteopontin, another ECM molecule expressed after demyelination, is also redundant for remyelination (Zhao et al., 2008). In contrast to stimulation of OPC proliferation by astrocyte-derived cFn, which promotes recovery, persistent expression of Fn variants in pathology mediates failure of tissue regeneration (Stoffels et al., 2013b). In particular the EIIIA domain is involved in adverse remodelling after tissue injury (Muro et al., 2008; Kohan et al., 2010; Arslan et al., 2011; Hirshoren et al., 2013). In MS lesions, Fn assembles into aggregates, which is likely mediated by inflammatory factors. Astrocytes are an important source of Fn aggregates, and these Fn aggregates contribute to remyelination failure (Stoffels et al., 2013a). Since our findings indicate that astrocyte-derived cFn is a non-essential element for remyelination, developing therapeutic approaches that remove aggregates of Fn, inhibitory to remyelination, from MS lesions, represent a legitimate translational objective.

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Chapter 4

Fibronectin aggregates promote features of a pro-inflammatory phenotype in microglia and bone marrow macrophages

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ABSTRACT

Promoting endogenous remyelination in multiple sclerosis (MS) benefits from insights into inhibitory molecules that mediate remyelination failure. Fibronectin assembles into aggregates in MS, which impair oligodendrocyte differentiation and remyelination. Microglia and macrophages are required for complete remyelination, and normally switch from an M1 pro-inflammatory phenotype on demyelination to a supportive, M2 phenotype during remyelination. Here, we investigated effects of fibronectin aggregates on microglia and macrophage phenotypes.

Bone marrow macrophages and microglia from newborn rats were exposed to a) plasma fibronectin coatings, b) coatings of deoxycholate-insoluble fibronectin aggregates, c) interferon-γ (IFN-γ) treatment, as an inducer of the M1 phenotype, d) interleukin-4 (IL-4) treatment, to promote the M2 phenotype, or e) were left unstimulated on uncoated plastic. In line with an M2 phenotype, exposure of microglia to fibronectin aggregates enhanced microglia proliferation, as was also observed on IL-4 treatment. However, in resemblance to IFN-γ treatment, exposure to fibronectin aggregates induced an amoeboid morphology of microglia and macrophages, and stimulated phagocytosis by macrophages. Culturing microglia and macrophages on fibronectin aggregates did not affect mRNA expression of the pro-inflammatory cytokines tumor necrosis factor-α, interleukin-1β and interleukin-12, but neither was there any effect on the expression of the anti-inflammatory cytokines arginase-1, CCL-2 and CCL-7. However, coatings of fibronectin aggregates, but not plasma fibronectin, promoted nitric oxide expression by microglia and macrophages.

In summary, macrophages and microglia grown on aggregated fibronectin showed a distinct phenotype with several pro-inflammatory features, including enhanced levels of nitric oxide. Therefore, persistent expression of fibronectin aggregates in MS lesions may maintain features of the M1 phenotype in microglia and macrophages, thereby possibly impairing remyelination.

INTRODUCTION

Multiple sclerosis (MS) is a chronic central nervous system (CNS) disease, of which inflammation, demyelination and neurodegeneration are major pathological features (Trapp et al. 1998, Barnett, Prineas 2004, Lassmann, Bruck & Lucchinetti 2007). Myelin regeneration (remyelination) by oligodendrocyte progenitor cells (OPCs) (Zawadzka et al. 2010) is apparent in early stages of MS (Patrikios et al. 2006). However, remyelination fails in chronic demyelinated lesions despite a relative excess of OPCs in the majority of MS lesions (Wolswijk 1998, Wolswijk 2002, Kuhlmann et al. 2008). Chronic demyelination correlates to neurological disability in MS (Trapp, Ransohoff & Rudick 1999). Hence, promoting endogenous remyelination is an attractive therapeutic strategy for structural recovery of MS lesions that will benefit from insights into mechanisms of remyelination failure.

Innate immune activity is an important feature of MS, as signified by activation of resident microglia and invasion of macrophages from the circulation through the disrupted blood-brain barrier (Bruck et al. 1995, Carson 2002). Effector functions of microglia and invaded macrophages depend on their phenotype, of which two major categories can be discerned. First, the pro-inflammatory phenotype M1 is characterized by secretion of reactive oxygen species and pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-12. The M1 phenotype is induced *in vitro* by stimulation with interferon-γ (IFN-γ) or lipopolysaccharide (LPS). Conversely, the alternatively activated M2 phenotype involves predominantly anti-inflammatory properties, including expression of arginase-1, CC chemokine ligand (CCL)-2, CCL-7, the mannose receptor, and growth factors such as insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF) (M2a, M2c) (Gordon 2003, Martinez, Helming & Gordon 2009). The M2 phenotype results from stimulation with interleukin-4 (IL-4) or IL-13. Although the M1/M2 concept was originally defined for macrophages, microglia are capable of adopting similar phenotypes (Durafourt et al. 2012). After demyelination, microglia and macrophages acquire a predominantly pro-inflammatory M1 phenotype, which leads to an increase of phagocytosis as well as expression of, among others, TNF-α, IL-1β and inducible nitric oxide synthetase (iNOS). During later stages of remyelination, however, oligodendrocyte differentiation benefits from a switch to the M2 phenotype, characterized by expression of arginase-1 and the mannose receptor (Olah et al. 2012, Voss et al. 2012, Miron et al. 2013). Signals from the micro-environment largely determine the phenotype of microglia and macrophages (Zhang, Mosser 2008).

Demyelination alters the expression of extracellular matrix (ECM) molecules (van Horssen, Dijkstra & de Vries 2007, Zhao et al. 2009), such as the glycoprotein fibronectin (Fn). Fn is transiently expressed as a dimer in demyelinated lesions (Zhao et al. 2009, Stoffels et al. 2013), resulting from plasma leakage across the blood brain barrier (Sobel, Mitchell 1989, van Horssen et al. 2005), and synthesis by local cells, including astrocytes (Stoffels et al. 2013, Hibbits et al. 2012). However, in MS lesions, Fn assembles into stable aggregates (aFn), which is likely mediated by ongoing inflammation, and aFn impairs oligodendrocyte differentiation and remyelination (Stoffels et al. 2013). Current evidence suggests that soluble dimeric plasma Fn (pFn) promotes several pro-

inflammatory functions of microglia (Milner, Campbell 2003, Milner et al. 2007, Goos et al. 2007, Ribes et al. 2010, Summers, Kielty & Pinteaux 2009). Given that remyelination likely benefits from a switch between a pro-inflammatory, M1 phenotype of microglia/macrophages on demyelination, to a M2 phenotype at later stages of remyelination (Miron et al. 2013), we investigated here how aFn affects microglia and macrophage phenotypes. Our data reveal that deposited fibronectin aggregates induce a distinct phenotype of microglia and bone marrow macrophages, supporting several pro-inflammatory features such as an amoeboid morphology and NO production. In this way, by sustaining distinct pro-inflammatory features of microglia and macrophages, aFn may further contribute to remyelination failure in MS.

MATERIAL AND METHODS

Cell culture

Microglia. Mixed glial cultures were derived from the cerebrum of newborn Wistar rats (P0-P2, Harlan, NL) and cultured in T75 flasks (Nunc, Naperville, IL) in Dulbecco's modified Eagle medium (DMEM; Gibco, Paisley, UK) containing 10% fetal-calf serum (FCS, Bodinco, Alkmaar, NL) and antibiotics (Life Technologies, Paisly, UK) for 10-12 days as described (McCarthy, de Vellis 1980, Bsibsi et al. 2012). To obtain shake-off microglia, flasks were mechanically shaken on an orbital shaker for 1 h at 150 rpm, after which the supernatant was centrifuged for 10 min at 1000 rpm. Cell pellets were resuspended in microglia medium (DMEM (Gibco) containing 10% Fn-free FCS (see below), antibiotics (Life Technologies) and rat recombinant macrophage colony-stimulating factor (M-CSF; 0.01 µg/ml, Peprotech, Rocky Hill, NJ) to ensure maturation of the immature neonatal microglia (Santambrogio et al. 2001). Shake-off microglia were cultured for 4 days at 37°C on 10 cm dishes (1,5-2.0 x 10^6 cells/dish, Corning, Lowell, MA). Subsequently, mixed glial culture flasks were shaken overnight at 240 rpm, 37°C and floating OPCs were purified by differential adhesion on 10 cm dishes (Greiner Bio One, Alphen aan den Rijn, NL), with the cells adhering to the bottom of the dish being in vast majority microglia. These differential adhesion microglia were cultured in microglia medium for 3 days at 37°C. Both microglia cultures, obtained from shake-off and differential adhesion, were pooled at the start of the experiments. At this stage, microglia cultures were typically > 95% pure, with approximately 4% astrocytes and >1% oligodendrocyte lineage cells as assessed by cell specific immunocytochemistry (see below) for ionized calcium-binding adaptor molecule-1 (Iba1; Abcam, Cambridge, UK), aldehyde dehydrogenase 1, member L1 (Aldh1L1; Neuromab, Davis, CA) and Olig2 (Millipore, Billerica, MA) respectively.

Astrocytes. To acquire purified astrocyte cultures, the remaining astrocyte monolayer of the mixed glial culture flasks was trypsinized and passaged once before experimental use (Bsibsi et al. 2012). Regular immunocytochemistry for glial fibrillary acidic protein (GFAP; Millipore) was performed as described below to assure sufficient purity of the astrocyte cultures (>97%).

Macrophages. To obtain bone marrow macrophages, hind legs of newborn Wistar rats (P0-P2, Harlan) were dissected and the bone marrow cavity of femur and tibia flushed with macrophage

medium, containing Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco), 10% Fn-free FCS (see below), 1% sodium pyruvate (Gibco), 0.05 M beta-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and antibiotics (Life Technologies). After centrifuging suspensions for 10 min at 1000 rpm, pellets were resuspended in BMM medium containing M-CSF (0.01µg/ml; Peprotech) to differentiate myeloid progenitor cells towards macrophages (Martinez et al. 2006). Macrophages were incubated for 7-8 days at 37°C on 10 cm dishes (2.0 x 10^6 cells/dish) (Boltz-Nitulescu et al. 1987, Davis 2013). Typical BMM cultures contained > 95% macrophages, as assessed by immunocytochemistry (see below) for isolectin-B4 (IB4; Invitrogen, Breda, NL).

Stimulations. At the start of experiments, microglia or macrophages were gently scraped in appropriate medium without M-CSF, and plated for experiments on 24-well plates (Nunc; 50.000/well in 500 µL of appropriate medium for 24 h), 8-well Permanox chamber-slides (Nunc; 30.000/well in 400 µL of appropriate medium for 24 h, except NO assays: 100.000/well in 300 µL of appropriate medium for 24 h), or 6-well plates (Corning, 1.0 10^6/well in 2000 µL for 6 h). Wells were pre-coated with pFn or aFn (see below), and after 1 h at 37°C, cells in uncoated wells were stimulated with either rat recombinant IFN-γ (5 U/µl, Peprotech), rat recombinant IL-4 (10 µg/ml, Peprotech) or left unstimulated.

Deoxycholate-insoluble fibronectin aggregates

Deoxycholate (DOC)-insoluble aFn was prepared from primary rat astrocytes (Stoffels et al. 2013). Astrocytes were cultured on 10 cm dishes (1.0 x10^6 cells/dish; Corning) in DMEM (Gibco) containing 10% heat-inactivated FCS (Bodinco) and antibiotics (Life Technologies), stimulated with polyinosinic:polycytidylic acid (pI:pC, 50 µg/mL, GE Healthcare, Freiburg, Germany) for 2 days at 37ºC to induce aggregation. After removal of astrocytes by water-lysis for 2 h at 37ºC, the remaining deposited astroglial matrices were scraped in ice-cold 2% DOC buffer (2% deoxycholate and Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany) in 20 mM Tris-HCl, pH 8.0) and further solubilized for 30 min on ice. To separate DOC-insoluble aFn from the suspension, centrifugion was performed for 30 min at 13300 rpm. Pellets were washed three times in phosphate buffer saline (PBS) followed by resuspension in PBS using a syringe and 25 gauge needle. As a result, a solution of DOC-insoluble proteins was obtained from the astroglial matrix, containing predominantly aFn, and lacking other extracellular matrix glycoproteins, such as laminin, because these are not DOC-insoluble (Stoffels et al. 2013). The protein concentration was determined by Bradford's protein assay (BioRad, Hercules, CA) using bovine serum albumin (BSA) as a standard. To confirm the presence of aggregated Fn and the absence of dimeric Fn, Western Blot analysis on aFn preparations was routinely performed as described (Stoffels et al. 2013) To coat wells, either bovine pFn (Sigma-Aldrich) or DOC-insoluble aFn were applied for 3 h at 37°C, using 5 µg on 8-well Permanox chamber slides wells (Nunc), and 50 µg on 6-wells plate wells (Corning).

Fibronectin-free serum

To eliminate Fn from FCS (Bodinco), a Gelatin Sepharose 4B column (GE Healthcare) was used according to the manufacturer's instructions. Resulting Fn-free serum was filter-sterilized for cell culture using 0.2 µM Whatman filters (GE Healthcare) and stored at -20°C until use. The virtual absence of Fn from the filtered serum and presence of Fn in the eluate was confirmed using Western blot analysis (data not shown).

BrdU incorporation assay

Microglia or macrophages were allowed to incorporate 5-bromo-2-deoxyuridine (BrdU) (10 µM, Roche) for 24 h. Cells were fixed in 4% paraformaldehyde (PFA) for 20 min, and additionally fixed in 5% acetic acid in ethanol for 20 min. BrdU was detected using reagents from the BrdU Labelling and Detection Kit I (Roche) according to the manufacturer's instructions with the addition of Iba1 (Abcam; 1:500) and Alexa Fluor© 546-conjugated anti-rabbit antibody (Invitrogen; 1:500) for microglia or Alexa Fluor© 546-conjugated IB4 (Invitrogen; 1:500) for macrophages, and visualization of nuclei with DAPI (Sigma, 1 µg/mL). The numbers of BrdU-positive nuclei were blindly counted relative to the Iba1- or IB4+-positive cells (at least 150 cells per condition) from images captured with a Leica TCS SP8 Confocal Laser Scanning Microscope.

Immunocytochemistry

Microglia or macrophages were fixed with 4% PFA for 20 min. After 30 min of blocking with 4% BSA in PBS containing 0.1% Triton (Sigma-Aldrich), cells were incubated for 1 h with primary antibodies in 4% BSA. Primary antibodies used were against Iba1 (Abcam; 1:500), Olig2 (Millipore; 1:1000), Aldh1L1 (NeuroMab; 1:50), GFAP (Millipore; 1:500) or Alexa Fluor© 546-conjugated IB4 (Invitrogen; 1:500). Cells were washed three times with PBS and incubated for 25 min with appropriate Alexa Fluor©-conjugated secondary antibodies (Invitrogen; 1:500). Nuclei were stained with DAPI (1 mg/ml), and fluorescence mounting medium (Dako, Heverlee, Belgium) was added to prevent image fading. Images were analyzed using an Olympus Provis AX70 fluorescent microscope (Olympus, New Hyde Park, NY) or a Leica TCS SP8 Confocal Laser Scanning Microscope. For morphology analysis, the morphology of each cell was scored as being 'ramified', 'amoeboid' or 'other'. Microglia or macrophages were considered 'ramified' if the shape was elongated with processes, 'amoeboid' if swollen with few processes, and 'other' if the shape could not be classified as either 'ramified' or 'amoeboid'.

Real-time, quantitative polymerase chain reaction (real-time qPCR)

Microglia or macrophages were cultured for 6 h, after which total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. 500 ng of RNA was reversely transcribed using oligo $(dT)_{12\text{-}18}$ (500 µg/ml), 10 mM dNTP Mix, 0.1 M dithiothreitol (DTT), 5x first strand buffer and Moloney Murine Leukemia Virus Reverse Transcriptase

(M-MLV RT) (all from Invitrogen).

Real-time qPCR was performed using the Applied Biosystems 7900HT Real-Time PCR System. Each reaction contained 5 ng cDNA, 10 pM primers (listed in table 1) and ABsolute SYBR Green Rox mix (Thermo Scientific, Landsmeer, NL). No-template controls were performed to ensure that amplification was not a result of contamination with genomic DNA. Gene expression levels were analyzed using the $2^{-\Delta\Delta ct}$ method (Livak, Schmittgen 2001), with normalization against HMBS or GAPDH. Similar results were obtained for both housekeeping genes; graphs shown means from normalization against HMBS.

Phagocytosis assay

Microglia or macrophages were cultured for 24 h, after which fluorescein isothiocyanate (FITC) labeled latex beads (1µm, Polyscience, Eppelheim, Germany) were added at a dilution of 10:1 beads:cells. Phagocytosis of the beads was allowed to proceed for 1 h, after which cells were fixed in 4% PFA. Cells were incubated with DAPI (1 µg/ml) and Alexa Fluor 568-conjugated IB4 (Invitrogen; 1:500) in 4% BSA for 2 h, washed in PBS and mounted using FluorSave (Calbiochem Millipore, Amsterdam, NL). In a blinded manner, the numbers of phagocytosed beads per cell were counted for 100 cells per condition, taking also into account their morphology, using an Olympus Provis AX70 fluorescent microscope (Olympus, New Hyde Park, NY).

Table 1. Primer sequences used for real-time, quantitative PCR

	Sense	Antisense
TNF-alpha	ATGGGCTGTACCTTATCTACTC	GTATGAAATGGCAAATCGGCT
IL-1beta	GAAGAATCTATACCTGTCCTGTG	TCTTTGGGTATTGTTTGGGA
IL-12	CTTTGAAGAACTCTAGGTGG	CTTGAGGGAGAAGTAGGAATGG
Arginase-1	ATATCTGCCAAGGACATCGT	ATCACTTTGCCAATTCCCAG
CCL-2	CATCAACCCTAAGGACTTCAG	AAGGCATCACATTCCAAATCAC
CCL-7	GACCAATTCATCCACTTGCT	ACTGGTGATCTTTCTGTAACTC
HMBS	CCGAGCCAAGCACCAGGAT	CTCCTTCCAGGTGCCTCAGA
GAPDH	CATCAAGAAGGTGGTGAAGC	ACCACCCTGTTGCTGTAG

Nitric oxide assay

Microglia or macrophages were cultured for 24 h, after which the medium was collected. The medium was briefly centrifuged for 5 min at 1200 rpm to eliminate cells from the medium. Then, medium was added to a reagent of 0.01% N-(1-Naphthyl)-ethylenediamine dihydrochloride (Fluka Analytical, Sigma-Aldrich) and 0.001% sulfanilamide (Sigma-Aldrich) in 2M HCl. The absorbance was measured at 550 nm and the NO concentration determined using a standard curve of sodium nitrite (2000 µmol/L in H_2O, Fluka Analytical, Sigma-Aldrich) in 1:1 solution of ultrapure water (Millipore):culture medium.

Lactate dehydrogenase and MTT assay

To determine the cytotoxicity of DOC-insoluble fibronectin aggregates, microglia or macrophages were cultured in 24-wells plates (Corning) on different concentrations of aFn (2 µg, 5 µg or 10 µg), while cells cultured on uncoated plastic were used as control, and wells kept with only culture medium were used for background subtraction All conditions were prepared in triplicate. After 24 h, the medium (lactate dehydrogenase (LDH) assay) and cells [3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT assay] were analyzed as described (Stoffels et al. 2013). Briefly, the release of LDH into the medium was measured using a commercial LDH assay kit (Roche) according to manufacturer's instructions. The effect on cell viability was determined with an MTT assay, for which cells were incubated with MTT diluted in culture medium (0.5 mg/ml, Sigma-Aldrich) for 4 h. MTT-formazan crystals were collected in dimethyl sulphoxide and absorption was measured at 560 nm.

Statistical analysis

Data were analyzed using SPSS and GraphPad Prism (GraphPad Inc, California, CA) and are reported as mean ± standard error of the mean (S.E.M.) of 2-5 experiments. Results of the proliferation, morphology, real-time qPCR, LDH and MTT analyses are presented as a relative to the unstimulated cells (US, set to 100% in each independent experiment). For categorical variables (BrdU proliferation assay, morphology), logistic regression was performed to compare proportions between the different conditions. Other data with continuous variables were tested for normality using the Kolmogorov-Smirnov normality tests. Since distributions of these data sets were incompatible with a normal distribution, the Kruskal-Wallis test was performed to compare means of all conditions in these experiments (real-time qPCR, phagocytosis assay, NO expression, LDH and MTT assays). For all analyses, p-values below 0.05 were considered statistically significant.

RESULTS

Fibronectin aggregates and plasma fibronectin promote proliferation of microglia, but not of bone marrow macrophages

During CNS demyelination, microglia and infiltrating macrophages increase their numbers by proliferation (Remington et al. 2007), thereby presumably maximizing their effector functions. In addition, ECM proteins, such as pFn and vitronectin, are upregulated in demyelinated lesions (Zhao et al. 2009, Stoffels et al. 2013, Sobel, Mitchell 1989, van Horssen et al. 2005, Hibbits et al. 2012, Satoh, Tabunoki & Yamamura 2009), and provide signals to enhance proliferation of microglia via integrin β1 (Nasu-Tada, Koizumi & Inoue 2005). To assess whether aFn, which is typically present in MS lesions (Stoffels et al. 2013), also contributes to such an expansion, we analyzed the effect of coated aFn and pFn substrates on proliferation of microglia and macrophages, using a BrdU incorporation assay. To this end, microglia or bone marrow macrophages, both derived from neonatal rats, were cultured on either uncoated wells, pFn or aFn. This approach revealed that of Iba1-positive microglia

left unstimulated, on average 10 ± 1% incorporated BrdU. However, as shown in Fig. 1A, proliferation of microglia increased by approximately 2-fold when cultured on both pFn and aFn coatings. Importantly, to eliminate a potential contribution of pFn normally present in serum, microglia and bone marrow macrophages were cultured in Fn-depleted serum at all conditions. Furthermore, to represent the M1 and M2 phenotypes, cells were also stimulated with IFN-γ or IL-4, respectively (Gordon 2003). For microglia, IL-4 induced a significant increase in proliferation (approximately 2.5-fold), in line with previous observations (Suzumura et al. 1994), whereas IFN-γ hardly, if at all altered proliferation (Fig. 1A).

Proliferation of IB4-positive macrophages was prominent and 20% ± 4% of the unstimulated cells incorporated BrdU (US, Fig. 1B). When the cells were grown on pFn or aFn coatings, proliferation of the macrophages was not markedly altered relative to unstimulated cells (Fig. 1B), in contrast to the substantial increase in proliferation of microglia (Fig. 1A). In addition, IL-4 did not alter proliferation of macrophages compared to US, whereas IFN-γ significantly reduced their proliferation (Fig. 1B). Because the morphology of microglia and macrophages corresponds to their phenotype

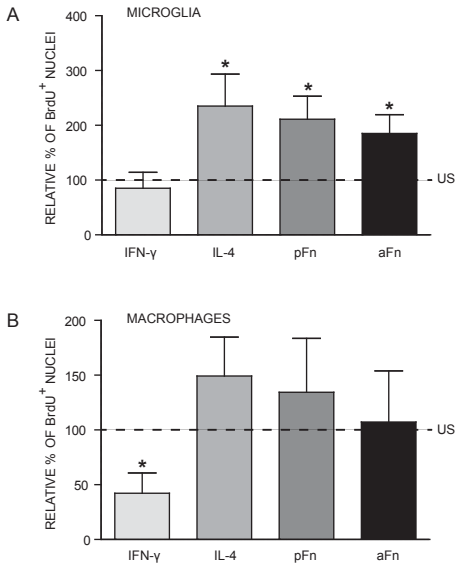

Figure 1. Fibronectin aggregates and plasma fibronectin promote proliferation of microglia, but not bone marrow macrophages.

A,B. Microglia (A) or bone marrow macrophages (B) were left unstimulated (US), cultured on plasma fibronectin (pFn) or fibronectin aggregates (aFn), or treated with interferon-γ (IFN-γ; 5 U/μl) or interleukin-4 (IL-4; 10 μg/ml). Subsequently, proliferation was determined by allowing the cells to incorporate BrdU for 24 h. BrdU-positive cells that also expressed Iba1 (microglia) or isolectin-B4 (macrophages) were counted. Compared to US microglia (10 ± 1% S.E.M.), proliferation was enhanced by culturing microglia on pFn and aFn substrates, resembling observation on IL-4 treatment. Conversely, compared to US macrophages (20 ± 4%, S.E.M, proliferation of macrophages on pFn and aFn coatings was not significantly affected. Bars represent mean percentages of cells incorporating BrdU, relative to US (set at 100%) from 3 independent experiments. Error bars show standard errors of the mean. Statistical analyses were performed using logistic regression for each experiment separately, representative statistical outcomes are summarized in the graph (* $p < 0.05$).

(Kreutzberg 1996, Vereyken et al. 2011), we next analyzed whether aFn similarly affects microglia and macrophage morphology.

Fibronectin aggregates and plasma fibronectin shift microglia and bone marrow macrophage morphologies towards amoeboid

M1 activated microglia and macrophages are characterized by a flat and rounded amoeboid morphology (Fig. 2A), whereas M2 polarized cells are predominantly ramified with elongated extensions (Fig. 2B) (Milner, Campbell 2003, Vereyken et al. 2011). To investigate whether aFn induces a morphology consistent with either phenotype, we performed immunocytochemistry for isolectin B4 (IB4), and blindly classified the morphology of the cells at each condition as either

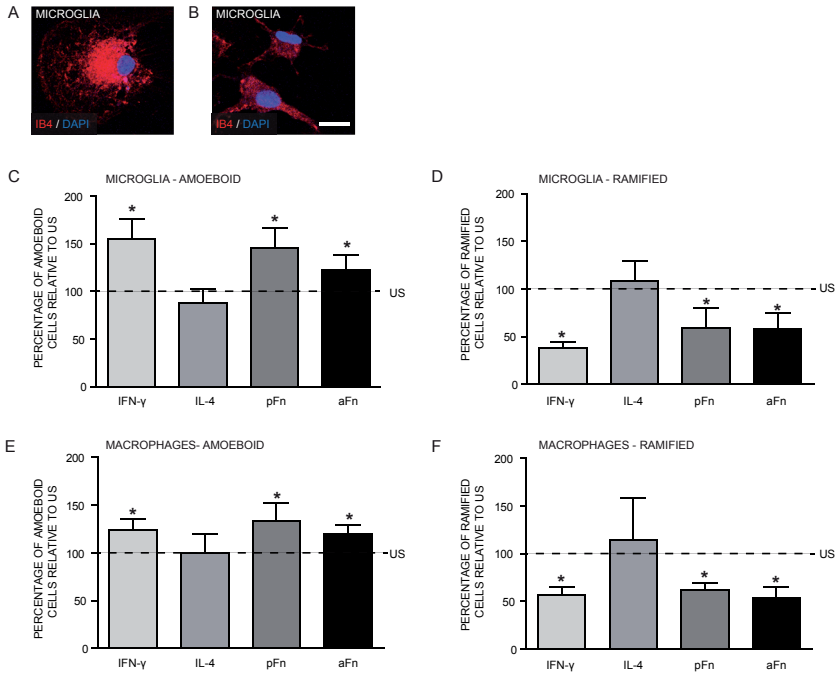

Figure 2. Fibronectin aggregates and plasma fibronectin shift microglia and bone marrow macrophage morphologies towards amoeboid.

A-F. Microglia (A,B,C,D) or bone marrow macrophages (E,F) were left unstimulated (US), cultured on plasma fibronectin (pFn) or fibronectin aggregates (aFn), or treated with interferon-γ (IFN-γ; 5 U/µl) or interleukin-4 (IL-4; 10 µg/ml). After 24 h, microglia (A,B,C,E) or macrophages (D,E) were immunostained with isolectin-B4, and morphologies of 100 cells per condition were scored as 'amoeboid' (A), 'ramified'(B) or 'other', with examples of US microglia shown in A and B. Dashed lines represent values of US cells set at 100% for each independent experiment. Note that the proportion of microglia and macrophages with an amoeboid morphology was promoted by IFN-γ treatment, pFn or aFn coatings relative to US cells (C-F). Bars represent mean values of each condition relative to US cells from 4 independent experiments. Error bars show the standard error of the mean. Statistical analyses were performed using logistic regression for each experiment separately, and representative statistical outcomes are summarized in the graph (* $p < 0.05$). Scale bar is 10 µm.

'amoeboid' (Fig. 2A), 'ramified' (Fig. 2B), or 'other'. The data revealed that both unstimulated microglia and macrophages predominantly displayed an amoeboid morphology. Hence, in unstimulated microglia, the relative contributions to the various morphological features were 55 ± 10% amoeboid, 30 ± 9% ramified and 15 ± 5% 'other'. In unstimulated macrophages, relative contributions were 51 ± 8% amoeboid, 31 ± 6% ramified and 18 ± 4% 'other'. These findings are in line with previous observations, which also revealed that culture conditions, and specifically culture serum, slightly activate microglia and macrophages towards the M1 phenotype (Adams et al. 2007, Ransohoff, Perry 2009). Following culture of the cells on pFn and aFn coatings, a significant increase, relative to unstimulated cells, was observed in the fraction of both microglia and macrophages displaying an amoeboid morphology, an effect similar to IFN-γ treatment (Fig. 2C-F). Simultaneously, the proportion of ramified microglia and macrophages decreased on pFn or aFn substrates, as well as following IFN-γ treatment (Fig. 2C-F). In contrast, and rather unexpectly (Wirjatijasa et al. 2002), exposure to IL-4 did not significantly change the morphology of microglia and macrophages relative to unstimulated cells (Fig. 2C-F). Importantly, the relative fractions of microglia and macrophages classified as being of 'other' morphology did not differ between all of the conditions examined (data not shown). Therefore, pFn and aFn shift the morphology of microglia and macrophages towards amoeboid, in line with the M1 phenotype. To further define the phenotype of microglia and macrophages, triggered on exposure to aFn, we next analyzed cytokine and chemokine mRNA levels, indicative of either M1 or M2.

Fibronectin aggregates and plasma fibronectin do not alter cytokine and chemokine gene expression representative for M1 or M2 by microglia and bone marrow macrophages

A typical feature of the M1 phenotype is the expression of pro-inflammatory cytokines, whereas M2 polarization generally induces the expression of anti-inflammatory factors (Gordon 2003). Here, we examined gene expression of TNF-α, IL-1β and IL-12 as representatives of the M1 phenotype. To mark anti-inflammatory M2 polarization, we analyzed mRNA levels of arginase-1, CCL-2 and CCL-7 (Gordon 2003, Martinez, Helming & Gordon 2009). Culturing microglia on either pFn or aFn did not change the expression of pro-inflammatory cytokines compared to unstimulated microglia (Fig. 3A). Surprisingly, pro-inflammatory cytokine gene expression was also not induced after IFN-γ exposure (Fig. 3A), in spite of the amoeboid morphology that was promoted at these same conditions (Fig. 2C,E). Anti-inflammatory gene expression was not enhanced by culturing microglia on pFn, whereas on aFn, CCL-7 expression was slightly increased (Fig. 3B). Further, IL-4 treatment markedly enhanced anti-inflammatory gene expression, most notably for arginase-1 and CCL-7 (Fig. 3B). In resemblance to the observations of microglia, culturing macrophages on pFn and aFn did also not induce a clear expression of either M1 or M2 cytokine gene signature (Fig. 3C,D). Rather, exposure to IFN-γ slightly upregulated the pro-inflammatory cytokine genes TNF-α and IL-12 (Fig. 3C), whereas IL-4 enhanced expression of arginase-1 and CCL-7 genes (Fig. 3D). Thus far, our descriptive analyses of morphology and mRNA signatures suggest a pro-inflammatory phenotype of either cell type on pFn and

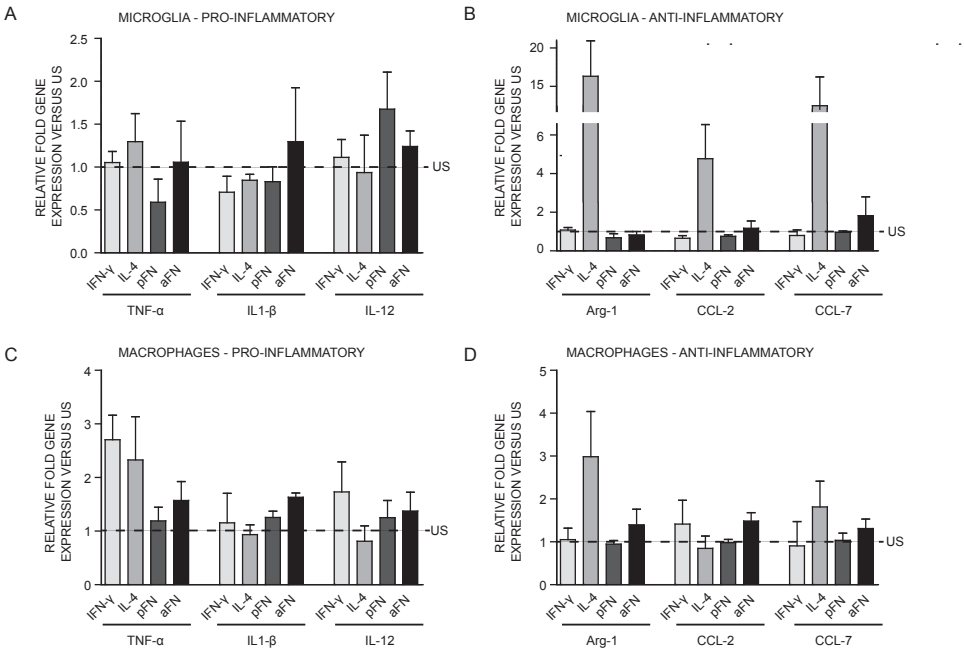

Figure 3. Fibronectin aggregates and plasma fibronectin do not alter cytokine and chemokine gene expression, representative of M1 or M2 phenotypes, in microglia and bone marrow macrophages.
A-D. Microglia (A,B) or bone marrow macrophages (C,D) were left unstimulated (US), cultured on plasma fibronectin (pFn) or fibronectin aggregates (aFn), or treated with interferon-γ (IFN-γ; 5 U/µl) or interleukin-4 (IL-4; 10 µg/ml) for 6 h, after which total RNA was extracted. Cytokine and chemokine gene expression levels were analyzed using quantitative real-time PCR against pro-inflammatory markers for the M1 phenotype (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-12 (IL-12)) (A, C) and anti-inflammatory markers for the M2 phenotype (arginase-1 (Arg-1), CCL-2 and CCL-7) (B, D) against HMBS (shown) and GAPDH (not shown, but yielding comparable findings). Bars represent mean expression levels versus US (set at 1 for each independent experiment) from 3 independent experiments, error bars show the standard error of the mean. Statistical analyses were performed using the Kruskal-Wallis test, followed by Dunn's Multiple Comparison Test, and did not reveal significant differences.

aFn, albeit without potent cytokine expression corresponding to M1. We therefore subsequently investigated functional properties of microglia and macrophages, grown on aFn coatings.

Fibronectin aggregates and plasma fibronectin selectively affect phagocytosis in microglia versus bone marrow macrophages

Phagocytosis by microglia and macrophages can be triggered by αvβ1 integrin-mediated signaling (Dupuy, Caron 2008, Ballana et al. 2011, Welser-Alves et al. 2011), with the integrin being a receptor for Fn. Here, we analyzed phagocytosis by measuring the numbers of fluorescently-labeled latex beads of 1 µm in diameter that were ingested over a period of 1 h by cells of either cell type (Chow, Downey & Grinstein 2004). Our data revealed that phagocytosis of latex beads in unstimulated

microglia amounted to 12 ± 1 beads/h. Culturing microglia on pFn or aFn did not markedly alter their phagocytotic activity (Fig. 4A). However, exposure of the microglia to IFN-γ or IL-4 reduced phagocytic activity of the cells by approximately 2-fold, showing a remaining ingestion activity of 5 ± 1 beads/h in both conditions (Fig. 4A).

Preliminary experiments further revealed that macrophages similarly displayed phagocytic activity, but these cells were less active in performing phagocytosis than microglia. Unstimulated macrophages ingested 4 beads/h (Fig. 4B), as compared to 10 beads/h by microglia (Fig. 4A). However, in contrast to microglia, phagocytotic activity of macrophages increased when the cells were cultured on pFn and aFn, or exposed to IFN-γ treatment. Similar to observations of microglia, exposure to IL-4 significantly reduced phagocytosis by macrophages (Fig. 4B).

Phagocytosis is considered a feature of M1-polarized microglia/ macrophages (Mosley, Cuzner 1996). Therefore, an enhanced uptake of beads may reflect the predominance of an amoeboid morphology. Indeed, on morphologically classifying each phagocytosing cell, a predominant amoeboid morphology was apparent for either cell type, i.e., 80 ± 6 % and 64 ± 13 % for microglia

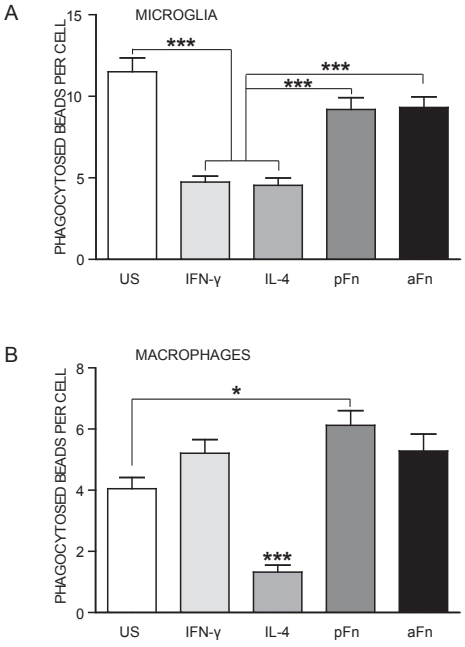

Figure 4. Fibronectin aggregates and plasma fibronectin promote phagocytosis by bone marrow macrophages, but not by microglia.

A,B. Microglia (A) or bone marrow macrophages (B) were left unstimulated (US), cultured on plasma fibronectin (pFn) or fibronectin aggregates (aFn), or treated with interferon-γ (IFN-γ; 5 U/µl) or interleukin-4 (IL-4; 10 µg/ml). Then, microglia and macrophages were allowed to phagocytose fluorescently-labeled latex beads for 1 h. The numbers of ingested beads were counted in isolectin-B4-positive cells. Bars represent mean numbers of phagocytosed beads. Error bars show the standard error of the mean. Representative graphs of duplicate experiments are shown. Statistical analyses were performed using the Kruskal-Wallis test, followed by Dunn's Multiple Comparison Test (* $p < 0.05$; ** < 0.01; *** < 0.001).

and macrophages, respectively. However, the numbers of beads phagocytosed per cell by amoeboid microglia did not significantly differ from the numbers of beads phagocytosed per cell by microglia of ramified or intermediate morphologies (Kruskal-Wallis test). In contrast, ramified macrophages phagocytosed significantly less beads on average than macrophages of amoeboid or intermediate morphologies ($p < 0,01$; Kruskal-Wallis test followed by Dunn's Multiple Comparison Test). Between the different conditions, however, the numbers of beads phagocytosed by ramified macrophages were similar to the numbers indicated above for each condition (Fig. 4B), when morphology was not taken into account. Therefore, differences in numbers of beads phagocytosed by microglia or macrophages do not merely signify dynamic changes that are reflected by their morphology. These results indicate that, in contrast to treatment with IFN-γ and IL-4, culturing microglia on aFn or pFn substrates does not markedly reduce phagocytosis. In addition, our preliminary data indicate that phagocytosis by bone marrow macrophages is promoted when the cells are grown on pFn and aFn coatings, in accordance with findings on IFN-γ treatment.

Fibronectin aggregates, but not plasma fibronectin, promote nitric oxide release by microglia and bone marrow macrophages

Because synthesis of reactive oxygen species is another important pro-inflammatory characteristic of microglia and macrophages (Gordon 2003) with relevance for MS (Smith, Lassmann 2002), we next examined NO release in the culture medium at 24 h after the different treatments. This approach revealed that whereas unstimulated microglia released little NO, culturing microglia on aFn tends to enhance NO levels in the medium (Fig. 5A). Similarly, IFN-γ treatment reproducibly increased NO release by microglia, and this enhancement was more prominent than when the cells were cultured on aFn coatings (Fig. 5A). Remarkably, culturing microglia on pFn did not enhance NO release as compared to unstimulated microglia (Fig. 5A).

For macrophages, NO levels in the culture medium displayed a very similar trend in response to the various conditions as those observed for microglia. A reproducible increase in NO release was triggered in macrophages grown on aFn, but not pFn coatings, while an approximate 4.5-fold increase in NO release was measured on exposure to IFN-γ (Fig. 5B). It should be noted that although unstimulated microglia and macrophages secreted fairly similar amounts of NO, in an absolute sense macrophages displayed an approximately 2.5-fold higher NO production on aFn coatings and after IFN-γ treatment (Fig. 5A,B).

To exclude the possibility that NO release by microglia and macrophages on aFn coatings may be a consequence of a cytotoxic effect induced by aFn, we performed LDH and MTT assays at different concentrations of aFn for both cell types. As shown in supplementary Fig. 1A and 1C, we found that LDH release by unstimulated microglia (0.50 mU/mL ± 0.05) and macrophages (1.1 mU/mL ± 0.25) did not differ from LDH release by cells of either type grown on aFn coatings. Also, microglia viability, as measured by MTT reduction, was comparable to the viability of unstimulated cells (supplementary Fig. 1B). Although viability of macrophages was slightly affected on aFn

(supplementary Fig. 1D), this reduction was not significant. Hence, aFn coatings do not induce significant cytotoxicity of microglia and macrophages, consistent with previous findings for OPCs (Stoffels et al. 2013). Therefore, these results indicate that aFn coatings promote a physiological relevant release of NO by microglia and macrophages.

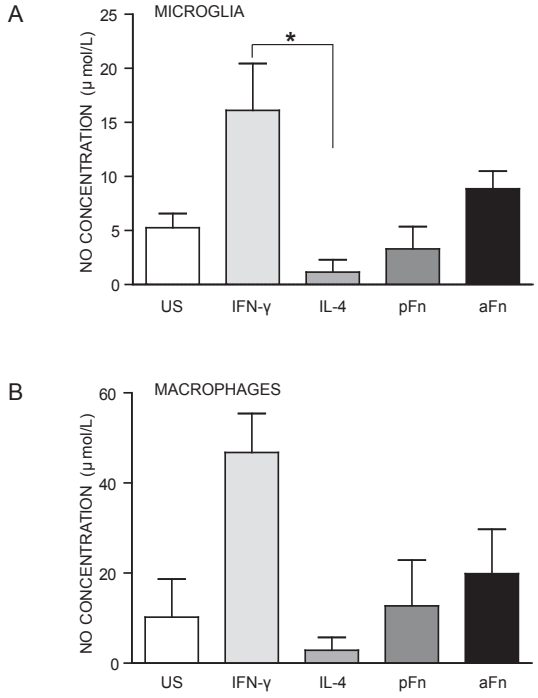

Figure 5. Fibronectin aggregates, but not plasma fibronectin, promote nitric oxide release by microglia and bone marrow macrophages.

A,B. Microglia (A) or bone marrow macrophages (B) were left unstimulated (US), cultured on plasma fibronectin (pFn) or fibronectin aggregates (aFn), or treated with interferon-γ (IFN-γ) or interleukin-4 (IL-4). Then, nitric oxide (NO) levels in the culture medium were analyzed as described in 'Materals and Methods'. Note that IL-4 reduced NO expression of microglia (A) and macrophages (B) relative to US cells, whereas IFN-γ and aFn enhanced NO synthesis. Bars represent mean NO concentrations for microglia (A) and macrophages (B) from 3 independent experiments, error bars show the standard error of the mean. Statistical analyses were performed using the Kruskal-Wallis test, followed by Dunn's Multiple Comparison Test (* $p < 0.05$).

DISCUSSION

In this study, we characterized microglia and macrophage phenotypes on coatings of Fn, an ECM protein that aggregates in MS lesions, thereby inhibiting remyelination (Stoffels et al. 2013). Our data revealed that pFn and aFn coatings enhanced proliferation of microglia, but did not significantly affect proliferation of bone marrow macrophages. Furthermore, both microglia and macrophages displayed a predominantly amoeboid morphology on pFn and aFn coatings. However, relative to unstimulated cells, these conditions did not lead to an induced expression of pro-inflammatory

or anti-inflammatory chemokine and cytokine genes. Rather, a slight but significant increase in phagocytosis by macrophages was observed when grown on pFn and aFn coatings. Moreover, in both microglia and macrophages, NO levels were enhanced on aFn, but not pFn substrates. Therefore, our data indicate that aFn promotes a distinct phenotype of microglia and macrophages with pro-inflammatory features.

Several microglia and macrophage inflammatory features were induced to a similar extent by both pFn and aFn, including a predominantly amoeboid morphology of macrophages, increased proliferation of microglia and enhanced phagocytosis by macrophages. Therefore, aggregation of Fn, which results from strong, non-covalent protein-protein interactions and is defined by DOC-insolubility (Ohashi, Erickson 2009), may represent a continuous state of pFn signaling, thereby activating several properties of microglia and macrophages. However, the aFn matrix also induced an additional effect compared to pFn, in that aFn enhanced NO release by microglia and macrophages. Possibly, this effect may be related to the potential presence of *cellular* Fn in aFn, which contains the alternatively spliced EIIIA and EIIIB domains that are absent from plasma derived pFn (Paul et al. 1986). The EIIIA domain is a ligand for the α9β1 receptor, and activation of this receptor promotes NO production in a human colon adenocarcinoma cell line (Gupta, Vlahakis 2009), indicating a possible mechanism for the aFn-mediated increase in NO levels. In previous studies, it has been reported that soluble pFn may also promote NO synthesis (Goos et al. 2007) as well as synthesis of pro-inflammatory cytokines, such as TNF-α (Goos et al. 2007, Ribes et al. 2010). Our seemingly opposing findings, which revealed that pFn coatings do not promote NO release and that both pFn and aFn coatings do not induce mRNA expression of pro-inflammatory cytokines, can likely be attributed to the different signaling properties of Fn coatings as opposed to those of soluble Fn. Immobilized Fn coatings, which probably better mimic the deposited Fn matrix in MS lesions than soluble Fn, enforce clustering of integrin receptors, and may also bind to different receptors, both integrins and others (Geiger et al. 2001). Indeed, the upregulation of, among others, TNF-α by soluble pFn is likely mediated via Toll-like receptor 4 (TLR-4) (Goos et al. 2007, Ribes et al. 2010), whereas coatings of pFn and aFn do not activate TLR-4 (our unpublished observations). This may also explain why Fn did not markedly enhance phagocytosis by microglia in our studies, which is considered to be mediated via TLR-4 (Ribes et al. 2010). Furthermore it should be noted that we investigated relatively naïve microglia and macrophages, whereas priming of microglia and macrophages may enhance their responsiveness to Fn. For instance, LPS-primed microglia respond to soluble pFn by additionally increasing IL-1β production (Summers, Kielty & Pinteaux 2009).

Microglia and bone marrow macrophages are distinct cell types (Graeber 2010), but their effector functions in MS are typically considered to be similar, as further enforced by the absence of a marker to distinguish both cell types (Rawji, Yong 2013). To clarify whether both cell types respond similarly to aFn, we directly compared activation profiles from microglia and bone marrow macrophages obtained in parallel from the same pool of neonatal rats. Although microglia and macrophages responded similarly with respect to morphology, NO synthesis and cytokine gene expression,

several differences were also apparent. First, microglia displayed more prominent phagocytotic activity than macrophages, with unstimulated microglia phagocytosing approximately 2.5-fold more beads than unstimulated macrophages. Potent phagocytosis by microglia is in line with previous reports (Durafourt et al. 2012, Mosley, Cuzner 1996) and likely sustains the notion that phagocytosis by microglia occurs without priming in CNS physiology (Schafer et al. 2012, Sierra et al. 2013). In addition, macrophages enhanced pro-inflammatory cytokine gene expression more readily on IFN-γ treatment than microglia, whereas microglia responded to IL-4 with a much greater increase of anti-inflammatory gene expression. Together with a more pronounced NO release by macrophages on IFN-γ and aFn, these findings support the concept that macrophages are immune mediators per se, whereas microglia display more plasticity with diverse, non-immunological roles in the healthy brain (Graeber 2010).

The overall effects of microglia and macrophage activation by aFn on remyelination remain to be established. Phagocytosis of myelin debris by macrophages is thought to promote remyelination (Kotter et al. 2006, Takahashi et al. 2007), but phagocytosis of neuronal debris can also enhance myelin and axonal destruction by the adaptive immune system (Huizinga et al. 2012). Similarly, NO has favorable effects on immune activation in MS, but also mediates oligodendrocyte and myelin injury (Smith, Lassmann 2002). In general, however, the early phase of remyelination benefits from M1 polarized microglia/ macrophage activity, whereas oligodendrocyte differentiation and myelin production are promoted by a switch to the M2 phenotype during later stages (Miron et al. 2013). Hence, transient Fn expression on demyelination (Zhao et al. 2009, Stoffels et al. 2013) likely contributes to the temporal M1 phenotype of microglia and macrophages, whereas the persistent presence of aFn in MS may prevent the necessary switch to an M2 phenotype. Indeed, in active MS lesions, macrophages are predominantly M1 polarized, although a major subset is also of an intermediate activation status (Vogel et al. 2013). In this way, aFn, among other factors, may promote unfavorable microglia and macrophage polarization. In addition to directly inhibiting OPC differentiation (Stoffels et al. 2013), this may represent an indirect mechanism for how aFn impairs remyelination.

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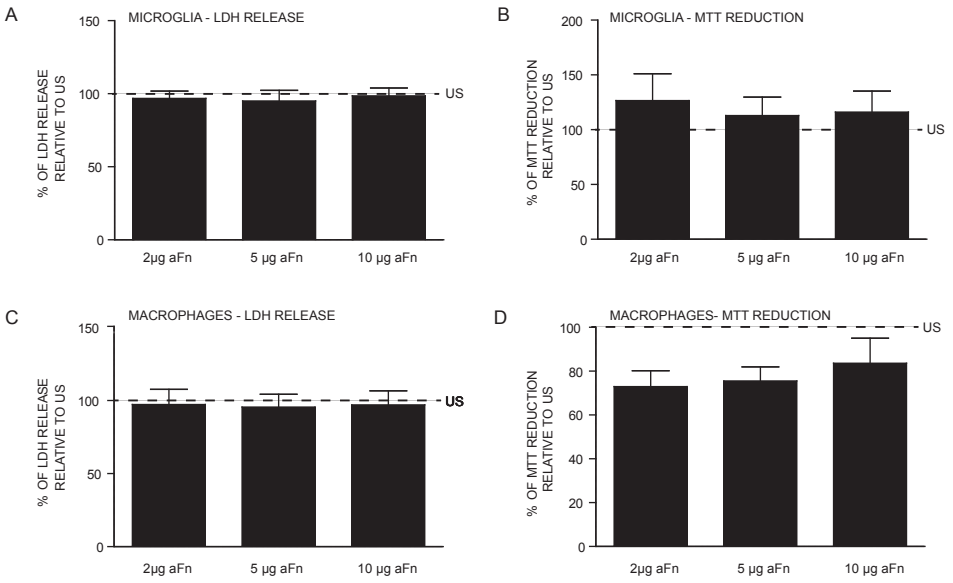

Supplementary figure 1. Fibronectin aggregates do not induce cell cytotoxicity as determined by lactate dehydrogenase release and an MTT assay.

A-D. Microglia (A) or bone marrow macrophages (B) were left unstimulated (US) or cultured on fibronectin aggregates (aFn; 2 µg, 5 µg or 10 µg respectively) for 24 h, followed by measurements of lactate dehydrogenase (LDH) release in the culture medium (A,C) and of MTT reduction (B,D). Bars represent means, error bars show the standard error of the mean. Statistical analyses were performed using the Kruskal-Wallis test, followed by Dunn's Multiple Comparison Test, and did not reveal significant differences.

Chapter 5

Fibronectin in tissue regeneration: timely disassembly of the scaffold is necessary to complete the build

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ABSTRACT

Tissue injury initiates extracellular matrix molecule expression, including fibronectin production by local cells and fibronectin leakage from plasma. To benefit tissue regeneration, fibronectin promotes opsonization of tissue débris, migration, proliferation and contraction of cells involved in the healing process, as well as angiogenesis. When regeneration proceeds, the fibronectin matrix is fully degraded. However, in a diseased environment, fibronectin clearance is often disturbed, allowing structural variants to persist and contribute to disease progression and failure of regeneration. Here, we discuss first how fibronectin helps tissue regeneration, with a focus on normal cutaneous wound healing as an example of complete tissue recovery. Then, we continue to argue that, although the fibronectin matrix generated following cartilage and central nervous system white matter (myelin) injury initially benefits regeneration, fibronectin clearance is incomplete in chronic wounds (skin), osteoarthritis (cartilage) and multiple sclerosis (myelin). Fibronectin fragments or aggregates persist, which impair tissue regeneration. The similarities in fibronectin-mediated mechanisms of frustrated regeneration indicate that complete fibronectin clearance is a prerequisite for recovery in any tissue. Also, they provide common targets for developing therapeutic strategies in regenerative medicine.

Fibronectin – elements of the scaffold

Fibronectin (Fn) is a high molecular weight glycoprotein, that consists of three types of repeating amino acid units, named type I, type II and type III repeats (Fig. 1). The structure of Fn depends on whether it is secreted in plasma or synthesized by resident cells. Plasma Fn (pFn) is produced by hepatocytes, and present in human blood at a concentration of 300 µg/mL (Nishinarita et al. 1990, Goos et al. 2007). Cellular Fn (cFn) contains the alternatively spliced extra domain A (EDA)

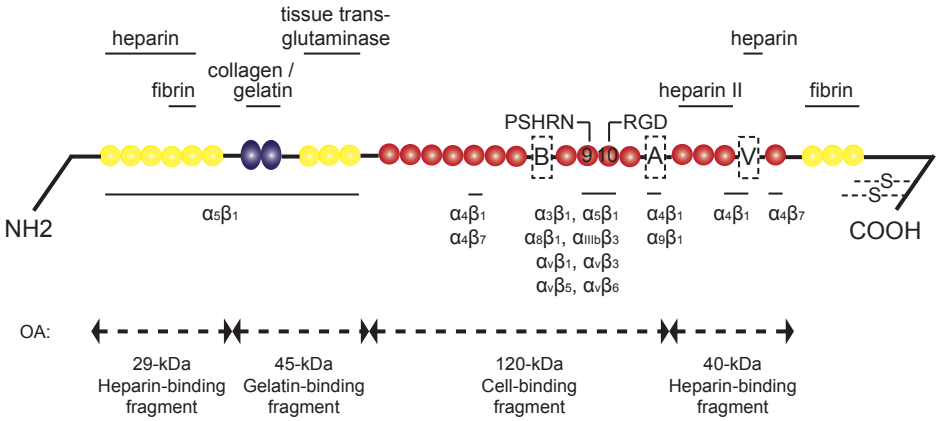

Figure 1. Structure of fibronectin and major fibronectin fragments that are generated in osteoarthritis. The yellow circles represent type I, the blue elipses type II and the red circles type III repeats. The alternative splice variants are referred to as 'A' for the EDA/EIIIA domain, 'B' for the EDB/EIIIB domain, and V for the V-region. Protein interaction sites are depicted above the linear structure, integrin binding sites below. PSHRN and RGD refer to these specific fibronectin domains. Four main fibronectin fragments with catabolic potential in osteoarthritis (OA) are shown under the double headed arrows. The arrows correspond to the cleaving sites of these fragments. Adapted from (Pankov, Yamada 2002, Sofat 2009, Schwarzbauer, De Simone, 2011).

and/or extra domain B (EDB) (nomenclature for humans; for rodents: EIIIA and EIIIB). In addition, a third alternatively spliced domain, the IIICS domain (for rodents: the V-region), can be included, but regulations for its inclusion haven't been fully discovered yet. pFn and cFn are secreted as a dimer, in which both subunits do not have to contain the same alternatively spliced variants. Physiological Fn monomers and dimers will hereafter be referred to as 'native Fn'. The main Fn receptors comprise a variety of integrin receptors (Fig. 1) (Plow et al. 2000). In addition, Fn binds other extracellular matrix (ECM) molecules, including heparin, collagen and fibrin, and together these protein networks form the ECM (Pankov, Yamada 2002).

The main function of Fn is to serve as a scaffold for cell adhesion and migration, thereby also regulating cell proliferation and differentiation (Pankov, Yamada 2002, Mosher 1988). These functions are supported by a variety of small proteins, such as growth factors, when they accumulate in the Fn network, increasing their concentration locally. Hence, these small molecules can be regarded as 'the builders' on a scaffold of ECM including Fn, although Fn itself also stimulates

tissue regeneration. The Fn matrix is essential for normal embryonic development (George et al. 1993). In healthy adult tissue, Fn is expressed at low levels. Transient Fn (re)-expression by plasma leakage and synthesis from resident cells is a common 'default' response of tissue injury, ranging from skin wounds to joint inflammation (Scanzello, Plaas & Crow 2008) and myelin degradation (demyelination). Here, we discuss first how this temporary Fn matrix facilitates tissue regeneration, with a focus on normal cutaneous wound healing as an example of complete tissue regeneration. Next, we review how in osteoarthritis (OA) and multiple sclerosis (MS), clearance of the Fn matrix is disturbed, and contributes to failure of tissue regeneration via distinct mechanisms.

The fibronectin scaffold helps to rebuild tissue: focus on cutaneous wound healing

Wounds are defined as disruption of the normal anatomical structure and function of tissue. The wound healing process is the physiological response to wounding in any tissue. Therefore, when we discuss how Fn benefits cutaneous wound healing, these functions equally apply to other tissues, although detailed features vary between tissue types (Velnar, Bailey & Smrkolj 2009). In cutaneous wounds, regeneration involves a) haemostasis and inflammation to provide temporary closure of the defect, b) migration and proliferation of epithelial cells to replace the temporary seal, and c) maturation and remodelling of the new epithelium and angiogenesis (reviewed, among many others, in (Velnar, Bailey & Smrkolj 2009, Tonnesen, Feng & Clark 2000, Baum, Arpey 2005)). Fn is involved in each of these steps to a greater or lesser extent (extensively reviewed in: (Clark 1988, Colvin 1989, Midwood et al. 2006)).

On skin injury, a temporary Fn matrix ('the Fn scaffold') originates from plasma leakage and cellular expression. First, whole blood containing pFn leaks from the disrupted vessels and pFn is a major component of the subsequently formed haemostatic clot, although pFn is not essential for normal haemostasis (Sakai et al. 2001). Haemostatic thrombi are, however, more stable with pFn than without (Ni et al. 2003). The haemostatic clot provides the basis of a provisional matrix, which also contains fibrin and other plasma proteins. The provisional matrix secures additional haemostasis, and assists migration and proliferation of epithelial cells. The presence of pFn in this matrix is not essential for normal wound healing (Sakai et al. 2001), which is explained by compensatory actions of cFn. For as soon as a few hours (Sakai et al. 2001) after wounding, cells start to deposit Fn in the provisional matrix. Initially, mainly platelets secrete cFn (Sakai et al. 2001), followed by macrophages, then fibroblasts (Brown et al. 1993), and possibly endothelial cells (Takamiya et al. 2006). In addition, neutrophils express Fn mRNA at 24 hours (Takamiya et al. 2006), but are negative again at 2 days after skin wounding (Brown et al. 1993). Therefore, neutrophils also contribute to initial cFn expression early after wounding. Analogous to cutaneous wounds, a temporary Fn matrix is generated on cartilage damage (Miller et al. 1984) and myelin damage (demyelination) in the central nervous system (CNS) (Sobel, Mitchell 1989, van Horssen et al. 2005, Satoh, Tabunoki & Yamamura 2009, Hibbits et al. 2012, Stoffels et al. 2013). In these injuries, Fn leaks from plasma (Sobel, Mitchell 1989, van Horssen et al. 2005) and is secreted by resident chondrocytes in cartilage (Chevalier et al. 1996,

Chevalier, Groult & Hornebeck 1996), and resident astrocytes, microglia and endothelial cells in the CNS (Stoffels et al. 2013). Therefore, the generation of a temporary Fn scaffold with pFn and cFn is a common response to tissue injury.

Functions attributed to the transient Fn matrix include stimulation of: a) coating and ingestion (opsonization) from tissue débris by inflammatory cells, b) migration and proliferation of regenerating cells via chemo- and/or haptotaxis, and c) angiogenesis [for cutaneous wounds reviewed in: (Tonnesen, Feng & Clark 2000, Clark 1988, Midwood et al. 2006, Clark 2001)]. Fn functions are similar among different tissue injuries, but mechanisms are best described in cutaneous wound healing. Fibroblast migration in wound healing requires functional RGD, heparin II and cFn IIICS domains (Clark et al. 2003) (Fig. 1), and is promoted by EIIIA from cFn via β-catenin (Bielefeld et al. 2011) and integrin α9β1 for keratinocytes (Singh et al. 2004). Further, myofibroblast differentiation is stimulated by EIIIA (Serini et al. 1998) via integrin α4β7 (Kohan et al. 2010). In fact, EIIIA is essential for normal wound contraction in mice as shown via EIIIA knockout (Muro et al. 2003), although in mice from a different genetic background, EIIIA knockout did not impair wound healing (Tan et al. 2004). Without EIIIB, mouse rib fractures heal normally, but more specific experiments are required to confirm its redundancy in cutaneous wound healing, especially since fibroblast proliferation and Fn matrix assembly *in vitro* are slightly reduced on EIIIB knockout (Fukuda et al. 2002).

Before tissue regeneration is completed, the Fn matrix is cleared. However, if Fn persists, this correlates to chronic failure of regeneration. In cutaneous wounds, analysis of wound fluid from human chronic venous ulcers showed persistence of Fn degradation products, possibly as a result of increased matrix metalloproteinase 9 (MMP-9) activity (Grinnell, Ho & Wysocki 1992, Moor, Vachon & Gould 2009). Certainly, failure of tissue regeneration is mediated by many factors, including changes in expression of growth factors, cytokines and matrix proteins as well as receptor expression patterns, and tissue oxygen levels (Falanga 2004, Widgerow 2012). Therefore, a structurally altered Fn matrix will only contribute to failure of regeneration in a complex interplay with changes in other factors, but nonetheless mediates tissue damage. Fn degradation products in chronic venous ulcers, for example, likely stimulate neutrophil degranulation (Wachtfogel et al. 1988), and EIIIA activates Toll-like receptor 4 (TLR4) on inflammatory cells. This TLR4 stimulation may help opsonization of tissue débris at first, but eventually results in chronic inflammation (Okamura et al. 2001, Gondokaryono et al. 2007). Interestingly, Fn fragments have also been implicated in OA disease progression, which will be discussed next.

Osteoarthritis: fibronectin fragments contribute to cartilage damage

OA is characterized by articular cartilage damage, resulting in joint destruction. The pathophysiology of OA is not fully understood. The current pathogenesis concept is based on increased cytokine and chemokine activation as a result of many factors, including ageing and chronic wear and tear on cartilage. In this concept, cytokines and chemokines contribute to protease production by chondrocytes, and inhibition of cartilage synthesis. This damages articular cartilage, and eventually

also joint synovium, ligaments, tendons and muscles (Loeser 2008, Edmonds 2009), causing pain and impairment of motility. The Fn matrix that is generated on the initial cartilage injury, is not completely degraded in OA. As a result, Fn fragments contribute to a) persistent local inflammation via innate immune system activation, and b) direct cartilage damage.

Local joint inflammation results from Fn persistence in several ways. First, after Fn is degraded into multiple fragments by proteases (Fig. 1), Fn binds the C1q component of the complement system (Bing et al. 1982, Carsons et al. 1988), likely resulting in chronic stimulation of leukocytes. Secondly, the fragment containing EIIIA stimulates TLR4, as discussed above in the context of complicated wound healing (Okamura et al. 2001, Lasarte et al. 2007). However, although the roles of EIIIA-TLR4 interactions in chronic wounds remain hypotheses based on *in vitro* studies, there is more evidence for the significance of this binding for joint damage. Injecting EIIIA-containing fragments into joints of mice results in joint swelling through the release of pro-inflammatory cytokines from mast cells (Gondokaryono et al. 2007). Therefore, although innate immune system activation by Fn initially facilitates tissue débris clearance on cartilage damage, non-degraded Fn fragments contribute to chronic synovial inflammation in OA.

Cartilage damage in OA (and in rheumatoid arthritis) is also mediated by Fn fragments via suppression of sulfated proteoglycans, and via stimulation of chondrocytes and synovial fibroblasts to secrete catabolic cytokines and MMPs. The four major characterized Fn fragments include a 29-kDa heparin-binding fragment, a gelatin-binding fragment, a cell-binding fragment and 40-kDa heparin-binding fragment (Yasuda 2006, Sofat 2009) (Fig. 1). These Fn fragments are present at high levels in synovial fluid from OA patients (Xie, Meyers & Homandberg 1992), and in human osteoarthritic cartilage (Zack et al. 2006). Fn fragments result from degradation of the Fn matrix, mediated by MMP-1, -3, -8 and -13 and by the aggrecanases ADAMTS-4 and -5 (Sofat 2009, Gendron et al. 2007, Zack et al. 2009). Native Fn has no adverse effects on cartilage, and low concentrations of Fn fragments show anabolic effects (Homandberg, Hui 1994). However, at higher concentrations Fn fragments stimulate cartilage chondrolysis *in vitro* (Homandberg, Meyers & Xie 1992, Yasuda, Poole 2002) via different effector molecules and different pathways.

First, Fn fragments contribute to release of pro-inflammatory cytokines. These include IL-1, TNFα and IL-6 in cultured human cartilage for the 29-kDa heparin-binding fragment (Homandberg et al. 1997), and IL-6, IL-8 (Pulai et al. 2005) and IL-7 (Long et al. 2008) in human articular chondrocytes for the cell-binding fragment. These cytokines subsequently stimulate MMP expression from chondrocytes, including MMP-1, -2, -3, -9 and -13 (Werb et al. 1989, Xie et al. 1994, Yasuda et al. 2003, Stanton, Ung & Fosang 2002), which enhance cartilage degradation. For example, chondrocytes release MMP-3 on stimulation with the 29-kDa heparin-binding fragment, but blocking antibodies to TNF-α, IL-1 and IL-6 suppress this release (Homandberg et al. 1997). Activation of some of these cytokines and MMPs is mediated by the nuclear factor-κβ (NF- κβ) transcription pathway (Pulai et al. 2005), whereas activation of others involves mitogen-acitvated protein kinase (MAPK) pathway activation (reviewed in: (Yasuda 2006)).

Secondly, Fn fragments induce MMP expression directly via cell surface receptors. For example, the 40-kDa heparin-binding fragment stimulates MMP release from chondrocytes via upregulation of NF-κβ through the phosphoinotiside-3-OH kinase (PI3K)/Akt pathway and the CD44 hyaluronan receptor (Yasuda et al. 2003, Yasuda 2011). Interestingly, the same heparin-binding fragment also binds TLR4 to initiate aggrecanase release from chondrocytes (Sofat, Robertson & Wait 2012). The cell-binding Fn fragment binds integrin α5β1 on chondrocytes and fibroblasts, which induces the secretion of MMP-13 and degradation of cartilage (Werb et al. 1989, Forsyth, Pulai & Loeser 2002, Homandberg, Costa & Wen 2002). This interaction requires reactive oxygen species as second messengers (Del Carlo et al. 2007). Although native Fn also binds to integrin receptors, native Fn stimulation does not cause a catabolic response from chondrocytes. This contrast can be explained by the hypothesis of 'Fn-integrin imbalance'. According to this hypothesis, Fn fragments alter normal Fn signals in chondrocytes by binding to distinct integrin receptors, but at the same time *not* binding to others. Therefore, chondrocytes perceive signals from altered clusters of integrins, and this initiates a catabolic response in OA (Werb et al. 1989, Huhtala et al. 1995, Peters, Loredo & Benton 2002). Further, Fn fragments expose cryptic binding sites, which also explains the altered signaling compared to native Fn.

Thirdly, besides stimulation of cytokine and subsequent proteinase (MMP) production, Fn fragments, such as the 29-kDa heparin-binding fragment, damage cartilage via suppression of cartilage matrix synthesis, including sulfated proteoglycans (Homandberg, Hui 1994, Homandberg, Meyers & Williams 1993). Also, the heparin-binding Fn fragment spanning the COOH-terminal induces an enhanced release of the free radical nitric oxide (NO) (Yasuda et al. 2004). Although most of the data discussed are generated *in vitro*, additional *in vivo* studies show that injection of Fn fragments into rabbit knee joints results in cartilage destruction and joint swelling, resembling OA in humans (Homandberg, Meyers & Williams 1993, Williams et al. 2003).

In order to reverse Fn fragment-mediated cartilage destruction in OA, and perhaps also rheumatoid arthritis, the following designs may be considered: a) prevention of Fn fragmentation, b) clearance of Fn fragments, and c) by-pass of harmful Fn fragment signals. Attempts at by-passing Fn fragment signals is, to our knowledge, the only approach to have been tested so far. Anti-oxidants, including N-acetylcysteine, glutathione and allopurinol, increase proteoglycan levels on Fn fragments as a result of a reduction of the catabolic cytokines TNF-α, IL-1 and IL-6 *in vitro* (Homandberg, Hui & Wen 1996a, Homandberg, Hui & Wen 1996b). Glucosamine and chondroitin sulfate mixtures also increase proteoglycan levels after Fn fragment administration to cultured cartilage (Homandberg et al. 2006). Despite these modest, favorable effects on the damage caused by Fn fragments, none of these agents are currently used in clinical treatments of OA. Another agent, hyaluronan, is in clinical use for OA, mainly because patients' pain and joint function can improve on this drug (Edmonds 2009). One of the underlying mechanisms for its benefit could comprise preventing the catabolic effects of Fn fragments, because on Fn fragment injection into rabbit joints, hyaluronan upregulates proteoglycan levels and improves histological disease

characteristics (Williams et al. 2003). In addition, after treatment with Fn fragments, hyaluronan promotes proteoglycan levels, and decreases NO levels in cultured human articular cartilage from OA patients (Kang et al. 1999, Yasuda 2010). Whether hyaluronan contributes to structural cartilage improvement in OA needs yet to be established in human patients.

Multiple sclerosis: fibronectin aggregates inhibit regeneration of myelin

MS is a chronic disease of the CNS. Although the pathogenesis is unknown, many factors are recognized to play a role in MS onset, including genetics and environmental factors, such as cigarette smoking, Epstein Barr virus infection and vitamin D levels (Ascherio, Munger & Lunemann 2012). Pathological hallmarks are CNS inflammation, myelin degeneration (demyelination) and axonal loss, which clinically reflect as neurological disability. Progression of MS occurs in distinct patterns, ranging from rapid accumulation of disability in primary and secondary progressive MS to episodes of fulminant inflammation and recovery in relapsing-remitting MS. On demyelination, regeneration of myelin (remyelination) is attempted by oligodendrocyte progenitor cells (OPCs), which can differentiate into myelin-forming oligodendrocytes. However, remyelination ultimately fails in MS, despite the presence of OPCs (Wolswijk 1998, Wolswijk 2002), leaving axons unprotected by myelin sheaths, and therefore vulnerable to further degeneration (Trapp, Nave 2008, Franklin, ffrench-Constant 2008, Franklin et al. 2012). Temporary, dimeric Fn expression occurs on demyelination, but Fn aggregates in MS lesions (Stoffels et al. 2013). Persistence of Fn, likely in the form of Fn aggregates, is involved in the pathology of chronic MS via a) (chronic) stimulation of inflammation, and b) direct inhibition of OPC maturation to oligodendrocytes (Fig. 2).

Inflammation in MS involves, among others, the entry of immune cells to the brain, and the pathological activation of CNS resident microglia. Leukocyte invasion to the brain in relapsing-remitting MS requires migration across the blood-brain barrier. The blood-brain barrier contains endothelial cells and astrocytes (Ballabh, Braun & Nedergaard 2004), expressing Fn (van Horssen et al. 2005, Man et al. 2009). In order to cross the blood-brain barrier, leukocytes express integrin α4β1, which mainly binds to vascular cell adhesion molecule 1 (VCAM1) (Rudick et al. 2012), but also to the CS1-peptide, which is a domain of Fn (a site within the V-region) (Fig. 1) on endothelial cells, or can be expressed independently of Fn on astrocytes (van der Laan et al. 1997, van der Laan et al. 2002). The α4β1-Fn CS1 interaction is blocked by natalizumab, an approved drug for relapsing-remitting MS (Man et al. 2009). Also, interferon β-1b, another drug for relapsing-remitting MS, inhibits the ability of T-lymphocytes to migrate via Fn on endothelial cells (Stuve et al. 1996). Therefore, Fn on endothelial cells contributes to leukocyte invasion in relapsing-remitting MS. Although cell migration and proliferation on Fn usually benefit tissue regeneration, these examples demonstrate that such physiological functions contribute to pathology under inappropriate circumstances. As an aside, stimulation of cell proliferation by Fn also becomes pathological in cancer metastases, when cancer cells invade new tissue and proliferate there (Kaplan et al. 2005, Malik et al. 2010, Reticker-Flynn et al. 2012). Further, Fn contributes to MS inflammation by instructing the CNS resident microglia and,

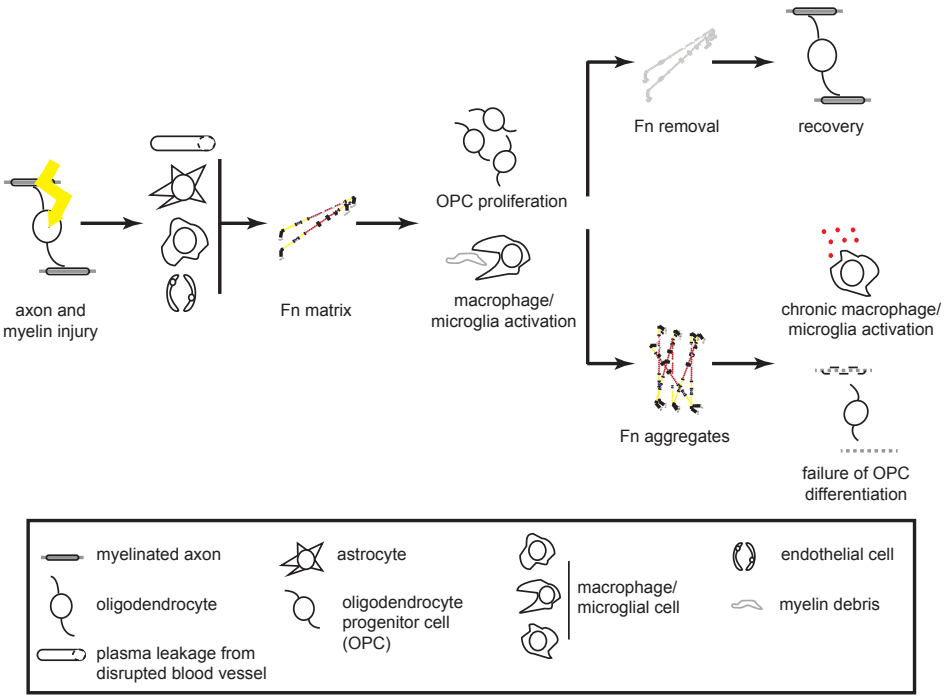

Figure 2. **On central nervous system injury in multiple sclerosis, improper fibronectin degradation is related to failure of remyelination and disease progression.** Myelin injury initiates fibronectin (Fn) expression from plasma leakage, and secretion by astrocytes, macrophages/ microglia and endothelial cells (depicted from top to bottom respectively). This Fn matrix promotes myelin regeneration via different mechanisms, including stimulation of oligodendrocyte progenitor cell (OPC) migration and proliferation.
Complete removal of the Fn matrix corresponds to remyelination, whereas Fn aggregates mediate failure of remyelination and axonal loss, likely via continuous macrophage/ microglia activation and impairment of OPC differentiation.

possibly, invaded macrophages. Fn interacts with integrin α5β1 on microglia to enhance MMP-9 secretion (Milner et al. 2007). Also, Fn may bind to TLR4 on microglia, as has been suggested for wound healing and OA before. Expression of TLR3 and -4 is upregulated in human MS lesions (Bsibsi et al. 2002), with TLR4 primarily localized to microglia (Bsibsi et al. 2002, Lehnardt et al. 2002). *In vitro,* pFn activates microglial cells to secrete pro-inflammatory cytokines, including IL-1β (Summers, Kielty & Pinteaux 2009), TNF-α, CXCL1, CCL3 and CCL5, and enhances phagocytosis by microglia (Ribes et al. 2010). Because these effects depend on the presence of MyD88, they likely result from TLR4 stimulation. Interestingly, in these studies, pFn was examined, suggesting that pFn contributes to TLR4-stimulation as well as EIIIA-containing Fn fragments (Okamura et al. 2001). Immune activated microglia likely facilitate remyelination in MS. For example, phagocytosis of myelin débris is necessary for complete remyelination (Kotter et al. 2006) and moderate inflammatory activity enhances remyelination (Li et al. 2005, Setzu et al. 2006). Also, macrophage/microglia activation by pFn is neuroprotective in traumatic brain injury (Tate, Garcia & LaPlaca 2007). However, TLR4

stimulation by LPS induces indirect loss of OPCs and oligodendrocytes, and neurodegeneration *in vivo* (Lehnardt et al. 2002, Lehnardt et al. 2003). Therefore, the overall effects of Fn-microglia interactions in MS remain to be established, as well as the effects of Fn aggregates.

Fn also directly affects OPCs. OPCs express a variety of integrin receptors during the (re) myelination process, including the Fn receptors αvβ1, αvβ3, αvβ5 and αvβ8 (Milner, ffrench-Constant 1994, Blaschuk, Frost & ffrench-Constant 2000, Zhao et al. 2009a). *In vitro,* coatings of pFn stimulate integrin αvβ1 to enhance OPC migration (Frost et al. 1996, Milner et al. 1996). In addition, αvβ3 integrin stimulation by physiological levels of platelet-derived growth factor A (PDGF-A) and Fn enhances OPC proliferation (Baron, Shattil & ffrench-Constant 2002). Further, integrin αvβ5 signalling is important for OPC differentiation (Blaschuk, Frost & ffrench-Constant 2000). These studies indicate that Fn promotes recruitment and subsequent proliferation of OPCs in the demyelinated area via αvβ1, αvβ3 and αvβ5 integrins, benefiting remyelination. However, *in vitro* myelin-formation of OPCs is impaired on pFn, mediated by β1-integrin signaling and mislocalized MMP-9 activity (Buttery, ffrench-Constant 1999, Siskova et al. 2006, Siskova et al. 2009). This impairment of OPC maturation initially plays a useful role in remyelination, because it allows for precise timing of the remyelination process (Franklin, ffrench-Constant 2008). However, as soon as OPC recruitment has been completed, αv integrin expression decreases (Zhao et al. 2009b) and the Fn matrix should be degraded, allowing OPCs to proceed to form myelin. Indeed, downregulation of Fn precedes remyelination on toxin-induced demyelination (Hibbits et al. 2012, Stoffels et al. 2013, Zhao et al. 2009a). In contrast, in chronic relapsing experimental autoimmune encephalomyelitis (cr-EAE), an animal model for relapsing-remitting MS, Fn aggregates in the lesion areas. Fn aggregates also persist in chronic demyelinated MS lesions, and inhibit CNS remyelination on toxin-induced demyelination *in vivo* (Stoffels et al. 2013). The mechanism for remyelination impairment needs yet to be established, but could comprise the perturbation of oligodendrocyte process outgrowth, myelin-membrane directed vesicular transport and membrane microdomain formation, as has been shown for pFn (Siskova et al. 2006, Siskova et al. 2009, Baron et al. 2003, Maier et al. 2005).

How Fn aggregates are formed in MS warrants further investigation. Organization of Fn into fibrils (fibrillogenesis) and, ultimately, assembly into a three-dimensional matrix is a well-balanced process during tissue development and regeneration [extensively reviewed in (Singh, Carraher & Schwarzbauer 2010, To, Midwood 2011b)]. Fn aggregation, as defined by deoxycholate (DOC)-insolubility, is likely the result of strong, noncovalent protein-protein interactions (Chen, Mosher 1996, Ohashi, Erickson 2009) (our unpublished observations), and participation of other extracellular proteins in this matrix (Ohashi, Erickson 2009). Fn aggregation may be appropriate during initial stages of tissue regeneration (Singh, Carraher & Schwarzbauer 2010, To, Midwood 2011b, McKeown-Longo, Mosher 1983). However, excessive Fn deposition and inappropriate remodeling contribute to scarring and fibrosis, and frustrate complete tissue regeneration (To, Midwood 2011b, Midwood, Williams & Schwarzbauer 2004). Under physiological circumstances, maintenance of the Fn matrix requires continuous Fn synthesis by cells (Sottile, Hocking 2002), but in MS, Fn mRNA levels were

undetectable in chronic demyelinated lesions, where Fn aggregates nonetheless persisted (Stoffels et al. 2013). This suggests that inappropriate remodeling, rather than continuous Fn deposition, is crucial for Fn aggregation in MS lesions. Fn remodeling into aggregates is likely mediated by self-assembly, interaction with binding sites on other proteins as well as with cellular receptors (mainly integrin receptors), and local enzyme activity (Singh, Carraher & Schwarzbauer 2010, To, Midwood 2011a, Ohashi, Erickson 2011). Because transglutaminase activity is proposed to be required for Fn aggregation (Geiger et al. 2001, Nelea, Nakano & Kaartinen 2008), and transglutaminase interactions with Fn are active in MS (van Strien et al. 2011), this enzyme is one of the factors that may contribute to Fn aggregation in MS, but this requires further investigation.

Concluding remarks: timely removal of the fibronectin scaffold is necessary to complete the build

In the development of therapeutic strategies for the promotion of tissue regeneration, there is much focus on the initiating mechanisms of specific diseases, for example via the identification of gene expression patterns relevant to disease onset. The rationale behind this approach is that to unravel the disease pathogenesis will likely provide targets for stopping tissue degeneration by its cause. Alternatively, a more pragmatic strategy is to tackle persistent factors in the injury environment that hamper regeneration. Fn is such a factor. This review illustrates similarities among the responses to injury in different tissues in the creation of a Fn matrix. Fn initially facilitates regeneration of skin, cartilage and myelin, mainly via stimulating the recruitment of inflammatory and regenerative cells. However, whereas Fn is totally removed before complete regeneration takes place, persistent structural variants contribute to failing regeneration in OA and MS. Mechanisms by which persistent Fn mediates regeneration failure differ between specific tissue types, but showed similarities, especially in their interaction with the immune system. Therefore, adequate clearance of the Fn matrix benefits regeneration, and incomplete degradation contributes to failure of tissue regeneration (Fig. 2).

The contribution of a residual Fn matrix in regeneration failure is not limited to the tissues that have been discussed in this review. For example, in myocardial infarction, the Fn matrix is necessary for myofibroblast recruitment and differentiation (Dobaczewski, Gonzalez-Quesada & Frangogiannis 2010), but on incomplete clearance of the matrix, the EIIIA-containing Fn mediates adverse cardiac remodeling (Arslan et al. 2011). Also, accumulation of Fn fragments occurs during intervertebral disc degeneration (Oegema et al. 2000), and further enhances disc degradation via stimulation of MMP expression (Greg Anderson et al. 2003, Xia, Zhu 2011). These examples further emphasize the benefit of complete degradation from the Fn matrix for tissue regeneration. This also underlines the importance of tightly-regulated dynamic ECM expression in general (Lu et al. 2011), especially because collagen fragments and persistence of tenascin-C also contribute to OA pathology (Chowdhury et al. 2010, Goh et al. 2010). Similarly, the high-molecular weight variant of hyaluronan, present in MS lesions, inhibits remyelination (Back et al. 2005).

In designing therapies to improve tissue regeneration, both the good and the bad sides of Fn can be taken into account. Taking advantage of the pro-regenerative properties of Fn, it can be attempted to speed up regeneration by exogenous administration of Fn. In designing such therapies, it is essential to consider the concept of 'dynamic reciprocity', which refers to the importance of well-balanced receptor and ligand signaling in time. In this concept, Fn administration can only add to healing if its (integrin) receptors are still upregulated (Widgerow 2012). Despite a potential mismatch between Fn and its receptors in chronic wounds, a modest additional benefit has been demonstrated for Fn-based therapies here. For example, after wounding the skin from obese diabetic mice (Livant et al. 2000), and also on rat peritoneal injury (Miyamoto et al. 2010), the PHSRN fragment from the 9th type III domain accelerates wound healing. This acceleration benefits from the physiological properties of Fn, including an increased fibroblast and keratinocyte adhesion and migration, wound contraction (Livant et al. 2000) and angiogenesis via integrin α5β1 on epidermal and endothelial cells (Feng, Mrksich 2004, Zeng et al. 2009). Similarly, pFn slightly accelerates wound healing in rats when topically applied onto skin wounds (Lariviere et al. 2003, Qiu, Kwon & Kamiyama 2007), and when injected after incisional wounding in a dose-dependent manner (Kwon, Qiu & Hiraon 2007). In patients with persistent corneal epithelial defects, topical application of pFn shows modest beneficial effects on healing (McCulley et al. 1993). Wound dressings, including Fn-based therapies, benefit healing in carefully selected wounds, and only in a subset of wounded patients (Boateng et al. 2008). Therefore, the therapeutic potential of exogenous Fn application could further be enhanced by selecting specific patient groups, such as wounded patients with diabetes mellitus (Qiu, Kwon & Kamiyama 2007). In addition, these therapies may be more effective when Fn domains are coupled to a) other supportive proteins, such as hylauronan (Ghosh et al. 2006), b) growth factors, such as PDGF (Lariviere et al. 2003) and hepatocyte growth factor (Okiyama et al. 2011), or c) glycoprotein hormones, such as erythropoietin (Kohan et al. 2010, Hamed et al. 2011). Finally, in another elegant approach, a fibrin/Fn matrix was designed, that could bind the growth factors PDGF, vascular endothelial growth factor (VEGF) and bone morphogenetic protein (BMP) to enhance healing of skin wounds from diabetic mice (Martino et al. 2011).

To overcome the detrimental properties of persistent Fn, multiple strategies are possible: a) ensuring proper Fn clearance, b) eliminating Fn structural variants once they emerge, and c) by-pass of harmful Fn signals. We briefly discussed therapeutic strategies in OA and MS that, among their other actions, by-pass Fn signals. By-passing strategies included hyaluronic acid (OA), natalizumab and interferon β-1b (MS), although these effects occur secondary to how the drugs were designed. These and the other approaches warrant further investigation. To accelerate our understanding, a multidisciplinary approach will be helpful, comparing good and bad sides of Fn and Fn therapies between tissues. This will expand our insight into how improper Fn clearance is mediated, and could be overcome. These insights will likely benefit therapeutic strategies that promote tissue regeneration.

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Chapter 6

Summary and Perspectives

SUMMARY

Multiple sclerosis (MS) is a chronic central nervous system (CNS) disease. The pathology of MS is characterized by inflammation, neurodegeneration and degradation of myelin (demyelination), the insulating membrane layer that enwraps axons and is produced by oligodendrocytes (Trapp et al. 1998, Barnett, Prineas 2004, Lassmann, Bruck & Lucchinetti 2007). Because myelin facilitates rapid nerve impulse conduction and protects axonal integrity, demyelination largely contributes to typical symptoms of MS, such as loss of motor and sensory functions. Indeed, chronic demyelination correlates to neurological disability in MS (Trapp, Ransohoff & Rudick 1999). Whereas regeneration of myelin (remyelination) by oligodendrocyte progenitor cells (OPCs) (Zawadzka et al. 2010) is apparent in early stages of MS (Patrikios et al. 2006), remyelination fails in chronic MS lesions. Therefore, structural therapeutic benefit is expected from promoting endogenous remyelination in MS. In order to effectively enhance endogenous remyelination, insights into mechanisms of remyelination failure in MS are pivotal. Endogenous remyelination likely fails in MS due to a) defective recruitment of OPCs to a subset of MS lesions (Williams et al. 2007, Boyd, Zhang & Williams 2013), and b) a failure of OPCs to differentiate into oligodendrocytes, which likely accounts for remyelination in the majority of MS lesions (Wolswijk 1998, Wolswijk 2002, Kuhlmann et al. 2008, Franklin, Ffrench-Constant 2008).

A major impediment for oligodendrocyte differentiation is the continuous expression of inhibitory signals, many of which originate from the altered microenvironment of OPCs in MS lesions **(chapter 1)**. Specifically, the extracellular matrix (ECM) is extensively remodeled in MS lesions (Sobel, Mitchell 1989, Gutowski, Newcombe & Cuzner 1999, van Horssen et al. 2005, van Horssen et al. 2006, Satoh, Tabunoki & Yamamura 2009), and aberrant signaling by several ECM proteins inhibits oligodendrocyte differentiation (Back et al. 2005, Lau et al. 2012). In this thesis, the role of the ECM glycoprotein fibronectin in remyelination was further investigated. Fibronectin is a dimeric protein, of which two major variants are discerned. Plasma fibronectin is continuously produced by hepatocytes and circulates in the plasma. Additionally, resident cells synthesize cellular fibronectin after tissue injury. Cellular fibronectin contains one or two alternatively spliced domains, EIIIA (nomenclature for rodents; EDA in humans) and EIIIB (EDB in humans), and may also contain the V-region (IIICS for humans). In demyelinated lesions, fibronectin is expressed (Sobel, Mitchell 1989, van Horssen et al. 2005, van Horssen et al. 2006, Satoh, Tabunoki & Yamamura 2009, Zhao et al. 2009, Hibbits et al. 2012), and *in vitro,* fibronectin inhibits oligodendrocyte maturation (Buttery, ffrench-Constant 1999, Maier et al. 2005, Siskova et al. 2006, Siskova et al. 2009).

In **chapter 2**, the expression patterns of fibronectin were examined in experimental models of demyelination and in MS lesions. This study revealed that an upregulation of fibronectin expression occurred after experimentally-induced demyelination and in demyelinated MS lesions. Whereas fibronectin likely leaked through the disrupted blood-brain barrier into demyelinated lesions, local cells also synthesized fibronectin. Major cellular sources of fibronectin were identified, including astrocytes, microglia/ macrophages and endothelial cells. Importantly, fibronectin

clearance correlated to complete remyelination, which is normally observed after toxin-induced demyelination. However, persistent expression of fibronectin corresponded to remyelination failure in chronic MS lesions. Moreover, in these inflammatory lesions, fibronectin was expressed in the form of deoxycholate-insoluble aggregates, rather than the dimers that were transiently expressed after toxin-induced demyelination. *In vitro*, these fibronectin aggregates were obtained from astrocytes by stimulating the cells with lipopolysaccharide (LPS), an immune mediator of the innate immune response. Indeed, fibronectin aggregates were only detected in demyelinated lesions on prolonged activation of the immune system, i.e. in active MS lesions and the chronic experimental autoimmune encephalomyelitis (cr-EAE) model. To analyze the functional consequences of fibronectin aggregates for oligodendrocyte differentiation, astrocyte-derived fibronectin aggregates were injected into toxin-induced demyelinated lesions, using saline injected lesions as controls. This demonstrated that fibronectin aggregates inhibit oligodendrocyte differentiation and impair remyelination. In further support of these findings, fibronectin aggregates were expressed at low level in remyelinated MS lesions ('shadow plaques').

Not only did astrocytes produce fibronectin aggregates, astrocytes also synthesized the transient, dimeric fibronectin matrix that preceded complete remyelination. Therefore, in **chapter 3**, the functional significance of transient fibronectin expression by astrocytes for complete remyelination was investigated, together with the significance of plasma fibronectin. Hereto, conditional knockout of fibronectin was induced from astrocytes, from plasma or from both astrocytes and plasma. Then, focal demyelination was created by injection of lysolecithin into the spinal cord ventral funiculus, and the course of remyelination studied versus littermate control animals injected with corn oil. These experiments revealed that astrocyte-derived fibronectin was important for OPC recruitment after demyelination, whereas plasma fibronectin was redundant. Additional *in vitro* studies showed that specifically the EIIIA domain from astrocyte-derived fibronectin stimulated proliferation of OPCs, but not their migration. The EIIIA domain was previously reported to increase cellular adhesion to fibronectin, thereby enhancing proliferation (Manabe, Oh-e & Sekiguchi 1999), but a role of EIIIA in adhesion of OPCs to fibronectin was not detected in our studies. Despite the contribution of astrocyte-derived fibronectin to proliferation of OPCs, remyelination completed normally in the absence of fibronectin from astrocytes. Therefore, the EIIIA domain of fibronectin from astrocytes promotes recruitment of OPCs, but is not essential for remyelination.

In addition to preventing differentiation of OPCs, fibronectin aggregates may also signal to other cells present in MS lesions. Indeed, soluble plasma fibronectin has been shown to promote several pro-inflammatory properties of microglia (Milner, Campbell 2003, Milner et al. 2007, Ribes et al. 2010). Innate immune activity from microglia and infiltrating macrophages benefits remyelination (Li et al. 2005, Kotter et al. 2005). In particular pro-inflammatory activity, characteristic for the M1 phenotype of microglia and macrophages, contributes to remyelination shortly after demyelination (Olah et al. 2012, Voss et al. 2012, Miron et al. 2013). However, at later stages, a switch in the microglia/ macrophage phenotype towards the anti-inflammatory, M2 phenotype stimulates

oligodendrocyte differentiation (Miron et al. 2013). Therefore, in **chapter 4**, it was investigated whether fibronectin aggregates induce a microglia/ macrophage phenotype, which may impair remyelination indirectly. In this study, coatings of fibronectin aggregates as well as plasma fibronectin were found to enhance proliferation of microglia, similar to the M2 phenotype provoked by interleukin-4 treatment. However, fibronectin aggregates and plasma fibronectin coatings also stimulated microglia and bone marrow macrophages to adopt an amoeboid morphology, similar to the M1 phenotype induced by interferon-γ. Although coatings of fibronectin aggregates did not promote expression of mRNA suggestive of the M1 or M2 phenotype, fibronectin aggregates, but not plasma fibronectin, did tend to promote nitric oxide expression by microglia and bone marrow macrophages, a feature also induced by interferon-γ treatment. This study suggests that fibronectin aggregates promote distinct pro-inflammatory features in microglia and macrophages. Functional consequences of this microglia and macrophage phenotype, induced by fibronectin aggregates, for remyelination remain to be established.

In studying the biology of remyelination, it has been proposed helpful to consider remyelination as the default response to myelin injury, bearing many similarities to regenerative processes occurring in other tissues of the body (Franklin, ffrench-Constant 2008). Along this line of thought, in **chapter 5**, the generation of a fibronectin matrix was reviewed as a common local response to tissue injury, resulting from injury to the skin (wounds), cartilage (osteoarthritis) and myelin (demyelination). This revealed common benefits of the fibronectin matrix for tissue regeneration, with important functions being the opsonization of tissue debris, as well as stimulation of migration, proliferation and contraction of cells involved in the healing process. However, in several diseases, clearance of fibronectin from the injured tissue environment is disturbed, allowing for the persistence of fibronectin structural variants. Fibronectin fragments persist in chronic wounds and osteoarthritis, whereas fibronectin aggregates remain present in MS lesions. Adverse functions of fibronectin structural variants for tissue regeneration were discussed, illustrating differences between tissue types, but also similarities. For instance, fibronectin structural variants tend to interact with the innate immune system, likely ensuring release of pro-inflammatory cytokines and nitric oxide in osteoarthritis and multiple sclerosis. The similarities in fibronectin-mediated mechanisms of frustrated regeneration indicate that complete degradation of not only fibronectin aggregates in MS, but also fibronectin structural variants in other diseases will contribute to tissue recovery. Also, identifying similar mechanisms of failed regeneration will likely provide common targets for developing therapeutic strategies in regenerative medicine.

PERSPECTIVES

This thesis identifies a dual role for fibronectin in remyelination of MS lesions, both helpful and adverse. To support remyelination, fibronectin promotes proliferation of OPCs and may contribute to transient pro-inflammatory polarization of microglia and bone marrow macrophages (chapter 5, Fig. 2). Future studies may identify additional beneficial functions of dimeric fibronectin. For example, fibronectin may coat myelin debris and thereby favor its phagocytosis (Clark 1988), which likely facilitates remyelination (Kotter et al. 2006). Also, fibronectin could favorably interact with other stimulatory factors in the demyelinated environment, most notably with growth factors such as transforming growth factor-β (TGF-β) (Fontana et al. 2005) and platelet-derived growth factor-AA (PDGF-AA) (Baron, Colognato & ffrench-Constant 2005), to increase their local availability. Although such beneficial functions of fibronectin are essential for embryonic development (George et al. 1993), results presented in this thesis imply that benefits of fibronectin are redundant for complete remyelination. Conclusive evidence on the redundancy of fibronectin for remyelination may be obtained in studies of remyelination after complete, conditional knockout of fibronectin, preventing fibronectin expression from any cellular source. Nevertheless, because a failure in recruitment of OPCs is apparent in a minority of MS lesions (Williams et al. 2007, Boyd, Zhang & Williams 2013), the theoretical possibility of therapeutically exploiting the beneficial functions of dimeric fibronectin in such lesions may be considered, paralleling strategies that promote wound healing (reviewed in chapter 5). However, in addition to major practical obstacles that would have to be overcome, such as identifying these lesions in patients and delivering fibronectin to inside these lesions, there is also the obvious risk of fibronectin aggregation. Hence, in attempts to take therapeutic advantage of beneficial functions of fibronectin in MS, it may be more feasible to target upstream regulators of fibronectin expression, which likely comprise, among other factors, TGF-β, retinoic acid and vitamin D (Borsi et al. 1990, Magnuson et al. 1991), in combination with strategies to prevent aggregation (further discussed below).

This thesis also demonstrates that aggregation of fibronectin in MS lesions impairs oligodendrocyte differentiation and remyelination (chapter 5, Fig. 2). Next to defective recruitment of OPCs (Williams et al. 2007, Boyd, Zhang & Williams 2013), the majority of MS lesions likely fail to remyelinate due to an impairment of oligodendrocyte differentiation (Wolswijk 1998, Wolswijk 2002, Kuhlmann et al. 2008, Franklin, ffrench-Constant 2008). In line with this hypothesis, several factors that promote oligodendrocyte differentiation have been identified, which may be relatively deficient in MS, such as agonists of the retinoic acid receptor-γ (Huang et al. 2011). Furthermore, a list of inhibitors of oligodendrocyte differentiation in MS lesions are now known (reviewed in chapter 1), to which fibronectin aggregates were added in this thesis. Although such inhibitors each have the ability to impair oligodendrocyte differentiation as such, potential interactions of their simultaneous presence in MS are unknown. Therefore, in future studies aimed at promoting endogenous remyelination, several hypotheses may be taken into account. Whereas it may be sufficient to overcome negative signals from a key inhibitor of oligodendrocyte differentiation in

the MS environment, it cannot be excluded that other hampering signals may then still prevail and frustrate remyelination. Additionally, after inhibitory signals have been overcome, the absence of stimulatory factors may constitute another obstacle before endogenous remyelination resumes. Nevertheless, it is predicted from this thesis that interference with the adverse effects of fibronectin aggregates will in any case (further) contribute to endogenous remyelination in MS, either independently or in combination with other therapies.

Theoretically, negative signals delivered by fibronectin aggregates could be counteracted by a) preventing fibronectin aggregation, b) clearing fibronectin aggregates from MS lesions, or c) masking cells for harmful fibronectin aggregate signals. Development of strategies to prevent fibronectin aggregation will benefit from a thorough understanding of how fibronectin aggregates are formed. Assembly of fibronectin in fibrils (fibrillogenesis) and, ultimately, a three-dimensional matrix is a complex, well-balanced process during tissue development and regeneration, during which fibronectin temporarily becomes DOC-insoluble (McKeown-Longo, Mosher 1983, Singh, Carraher & Schwarzbauer 2010, To, Midwood 2011). Conversion of dimeric fibronectin to fibronectin aggregates likely involves i) ongoing fibronectin synthesis by local cells, and ii) remodeling of the fibronectin matrix, which requires self-assembly, interactions with binding sites on other ECM proteins and with cellular receptors (such as integrin receptors), as well as local activity of several enzymes (Singh, Carraher & Schwarzbauer 2010, To, Midwood 2011, Sottile, Hocking 2002, Ohashi, Erickson 2011). In chronic MS lesions, fibronectin mRNA levels are downregulated, whereas fibronectin aggregates persist (chapter 2), suggesting that inappropriate remodeling mediates fibronectin aggregation in MS more dominantly than continuous fibronectin synthesis. To elucidate how fibronectin is remodeled, each of the required steps described above may be studied. In particular the enzyme transglutaminase may be of interest, because transglutaminase activity is likely required for fibronectin aggregation (Geiger et al. 2001, Nelea, Nakano & Kaartinen 2008) and this enzyme interacts with fibronectin in MS lesions (van Strien et al. 2010). Studies of aggregate formation by interactions between fibronectin and cellular receptors, which may be therapeutically blocked, also warrant investigation. In studying mechanisms of fibronectin aggregation in this way, it may be useful to take into account that not only fibronectin, but also other ECM components are extensively remodeled in the inflammatory MS environment. Many ECM proteins are permanently upregulated (Fawcett, Asher 1999, van Horssen, Dijkstra & de Vries 2007) and some are known to be structurally altered, for instance hyaluronan (Back et al. 2005). Together with cellular components, in particular reactive astrocytes, the remodeled ECM eventually forms a glial scar in MS lesions. The glial scar has long been recognized as an end-result of frustrated regeneration in a chronic lesion environment (Fawcett, Asher 1999), but surprisingly little is known about how the ECM is remodeled to form the glial scar. However, the interplay between ECM proteins is worthwhile addressing, among others because conformational changes in other ECM proteins may further facilitate fibronectin aggregation. For example, different conformations of tenascin-C exert distinct effects on fibronectin matrix assembly (To, Midwood 2010). Insights into glial scar formation may gain

from careful examination of adverse remodeling in other parts of the body with similar key players, such as pulmonary fibrosis, in which fibronectin is strongly involved (Muro et al. 2008). Also, master regulators of remodeling will likely be similar among tissue types, with an obvious candidate being TGF-β (To, Midwood 2011, Leask, Abraham 2004). In short, a better understanding of fibronectin and ECM remodeling may provide targets for interfering with fibronectin aggregation and also with glial scarring, thereby promoting regeneration. Appropriate timing of an intervention to prevent aberrant scarring will likely be pivotal.

In a second approach, therapeutic benefit may be achieved by clearing fibronectin aggregates from the MS environment. Fibronectin clearance is normally mediated by endocytosis, requiring β1 integrin and caveolin-1 (Shi, Sottile 2008), and is facilitated by local protease activity. Important proteases that mediate degradation of fibronectin are matrix metalloproteinases (MMPs), including MMP-3, -7, -9, -10 and -13, and members of the protease family 'a disintegrin and metalloproteinase with thrombospondin motifs' (ADAMTS), such as ADAMTS-5 (Lu et al. 2011). Expression of MMPs and ADAMTS, in turn, is regulated by other enzymes, including tissue inhibitors of metalloproteinases (TIMPs), and by the ECM. Indeed, several MMPs and TIMPs are upregulated after experimental demyelination (Skuljec et al. 2011). The importance of adequate proteolysis has become evident from, for example, MMP-9 null mice, in which experimental remyelination is impaired (Larsen et al. 2003), which could also reflect hampered clearance of fibronectin. In MS lesions, MMP activity is disturbed with a distinct upregulation of many MMP members (Agrawal, Lau & Yong 2008). Restoring a balance in MMP activity may favor fibronectin aggregate clearance. This would require a thorough understanding of the normal balance between proteases involved in degradation of fibronectin and the ECM, which is currently lacking (To, Midwood 2011).

Finally, inhibitory effects of fibronectin aggregates on remyelination could be overcome by blocking signals from fibronectin aggregates to cellular receptors on OPCs, microglia and macrophages. In this approach, fibronectin aggregate signals may be blocked at several levels, for example by interfering with receptor–fibronectin interactions, or by inhibiting adhesion molecules that mediate signals from fibronectin, such as glycosphingolipids associated with lipid rafts (Simons, Toomre 2000). A candidate approach could identify possible targets to overcome signals from fibronectin aggregates in this way. Possibly in contrast to preventing aggregation or clearing fibronectin aggregates, masking cells for the adverse signals of fibronectin aggregates may involve relatively few molecular players. As an additional advantage, blocking signaling by fibronectin aggregates may enhance endogenous remyelination regardless of the time at which blocking agents are administered, whereas the time window for successfully preventing fibronectin aggregation may be narrow. Hence, the most feasible option to overcome the impairment of remyelination by fibronectin aggregates may be to block their signals to OPCs and other cell types, such as microglia and infiltrated macrophages. Based on the studies presented in this thesis, it is predicted that such strategies to overcome adverse effects of fibronectin aggregates will contribute to endogenous remyelination of MS lesions.

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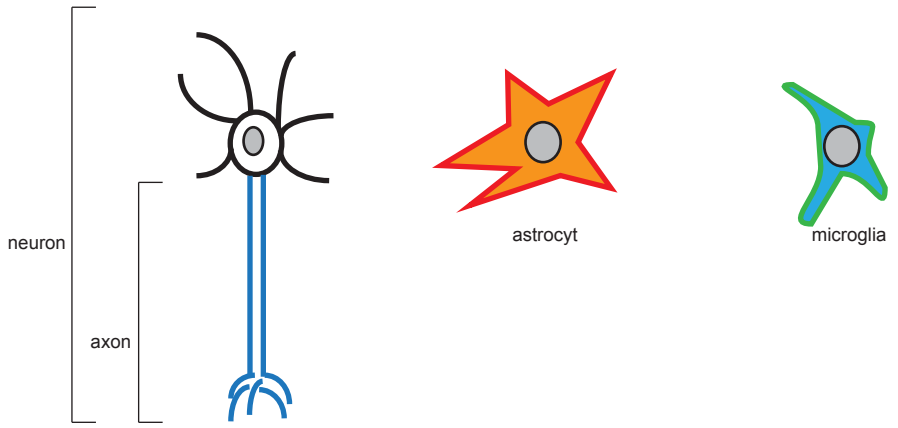
Nederlandse samenvatting

Als een salamander zijn staart verliest, groeit de staart vanzelf weer terug. Een sterk staaltje weefsel regeneratie, dat is voorbehouden aan reptielen en waar mensen alleen van kunnen dromen. Of... is dat zo? Misschien is het menselijk lichaam niet zo veerkrachtig als dat van een salamander, maar toch groeien ook onze weefsels tot op zekere hoogte terug na beschadiging. Overweeg dit eens, bijvoorbeeld: wanneer u, een gezond mens, zich in uw vingers snijdt, dan geneest de huid zichzelf, soms zelfs zonder litteken. Ook als een weefsel of orgaan beschadigt raakt door een ziekte, dan ziet men bij weefselonderzoek vaak vergelijkbare tekenen van een genezingsproces zoals zich dat afspeelt in een wond van de huid. Maar de genezing van een weefsel is doorgaans onvolledig indien de beschadiging onderdeel is van een ziekte. Misschien kunnen we proberen te begrijpen waarom de genezing van het weefsel in dergelijke gevallen niet slaagt, zodat we met gebruik van dergelijke kennis het lichaam een handje kunnen helpen en weefsel terug kunnen laten groeien. Een beetje zoals de staart van de salamander.

Vanuit deze invalshoek werd in dit proefschrift het genezingsproces van myeline, de vette isolatie rondom zenuwen (zie onder), bestudeerd in de ziekte multiple sclerose (MS), een ziekte van het centrale zenuwstelsel. Het centrale zenuwstelsel laat zich inderdaad vergelijken met een centrale; een drukke communicatiecentrale van de zenuwen van de hersenen en het ruggenmerg, die informatie uitwisselen met de rest van het lichaam. Gevoelens van warmte of knellende schoenen, het zien van letters, woorden en zinnen, het omslaan van een bladzijde; dergelijke informatie ontstaat in het centrale zenuwstelsel of wordt daarin voor een belangrijk deel verwerkt. Een patiënt met MS heeft plaatselijke beschadigingen ('laesies') aan het centrale zenuwstelsel, waardoor de warmte van een hete ovenschaal op de hand niet meer goed gevoeld wordt of een deel van een bladzijde niet gezien kan worden, bijvoorbeeld. In het begin van de ziekte zijn deze beperkingen vaak tijdelijk van aard en de laesies genezen gedeeltelijk, maar uiteindelijk krijgen veel MS patiënten steeds meer handicaps, die niet meer herstellen. Beschikbare therapieën kunnen het aantal aanvallen van MS dat optreedt iets verminderen, maar de voortgang van de ziekte niet geheel remmen. Ook kan ontstane schade niet geheel worden hersteld.

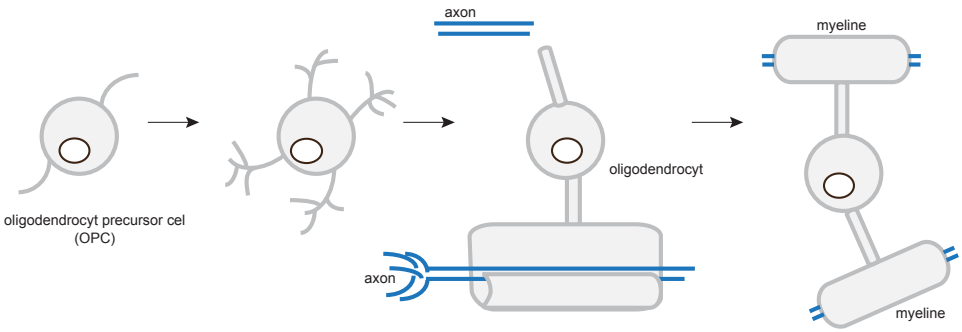
Om te begrijpen waarom in het zenuwstelsel van MS patiënten dergelijke beschadigingen niet genezen, is het zinvol te bestuderen uit welke cellen het zenuwstelsel is opgebouwd en hoe deze worden aangetast door de ziekte, zoals werd gedaan in hoofdstuk 1. De ene helft van het centrale zenuwstelsel bestaat uit zenuwcellen (neuronen; Figuur 1), de lange signaalcellen die vaak vergeleken worden met elektriciteitskabels, en die elektrische zenuwsignalen (zoals: 'uw hand is warm' of 'dit lees ik nu') communiceren tussen de hersenen en de rest van het lichaam. De andere helft bestaat uit glia cellen; een verzamelnaam voor vele verschillende celtypen. 'Glia' is afgeleid van het Griekse woord voor 'lijm' en inderdaad werd lang aangenomen dat glia cellen als belangrijkste functie hadden steun te bieden aan de neuronen. Maar de laatste tijd ontdekken we dat glia cellen ook functies vervullen naast het ondersteunen van neuronen. Voor dit proefschrift zijn de belangrijkste soorten glia cellen die werden bestudeerd: a) astrocyten; stervormige cellen (Figuur 1), waarvan de precieze functies nog onopgehelderd zijn, b) microglia (Figuur. 1); cellen die



Figuur 1. Enkele belangrijke cellen uit het centrale zenuwstelsel: een neuron, waarvan de lange uitloper 'axon' wordt genoemd; een astrocyt en een microglia cel.

zusjes zijn van de veelzijdige afweercellen 'macrofagen' buiten het zenuwstelsel, maar daarnaast waarschijnlijk ook een rol spelen in het aanpassen van neuronale netwerken, en c) oligodendrocyten en hun voorlopercellen, oligodendrocyt precursor cellen (OPCs) (Figuur 2). OPCs zijn kleine, ronde cellen met korte uitlopers. Tijdens de ontwikkeling van het zenuwstelsel groeien de uitlopers van de OPCs uit tot lange, brede membranen (vliezen), die zich tenslotte heel strak om de uitlopers van de neuronen ('axonen') heen wikkelen. Deze omhulsels rondom axonen noemen we 'myeline scheden' (Figuur 2). OPCs groeien uit tot oligodendrocyten onder invloed van een intern regelsysteem; een soort 'interne klok', die wordt aangestuurd door externe signalen van buiten de cel. Het myeline van de volwassen oligodendrocyt zorgt op deze manier voor elektrische isolatie van een groot gedeelte van het axon, zodat de zenuwcellen boodschappen (zoals 'til deze bladzijde op') sneller doorgegeven. Daarnaast beschermt myeline het axon en bespaart het axon mogelijk ook voedingsstoffen.

Een belangrijk onderdeel van de ziekte MS is dat myeline plaatselijk beschadigt ('demyelinisering'), hoewel nog niet duidelijk is hoe dat precies gebeurt. OPCs blijven verspreid door het volwassen zenuwstelsel en zouden daarom theoretisch nieuwe oligodendrocyten en nieuw myeline kunnen maken om de myeline schade in MS te herstellen. Dit proces heet 'remyelinisering'. In diermodellen is al aangetoond dat remyelinisering kan leiden tot functioneel herstel; het zenuwstelsel gaat weer beter werken en bijvoorbeeld de hete ovenschaal zou weer kunnen worden gevoeld. Inderdaad zien we ook in sommige MS laesies een nieuw, dun laagje myeline. Maar in de meeste MS laesies blijven we een groot aantal OPCs aantreffen, die niet uitgroeien tot volwassen oligodendrocyten en die geen nieuw myeline maken. Remyelinisering faalt dus in MS. Waarom slagen OPCs er meestal niet in nieuw myeline te maken? Het antwoord is niet geheel opgehelderd, maar het is waarschijnlijk dat zich in MS laesie, en dus de omgeving van OPCs, twee hoofdgroepen van obstakels laten onderscheiden:



Figuur 2. De oligodendrocyt precursor cel (OPC) groeit in enkele stappen uit tot een volwassen oligodendrocyt. Dit gebeurt onder invloed van een 'interne klok', die tevens wordt aangestuurd door externe signalen uit het milieu van de OPC. De volwassen oligodendrocyt maakt contact met axonen. Dan wikkelt het vettige vlies (membraan) van de oligodendrocyt zich strak om het axon. Zo ontstaat de myeline schede, die er onder andere voor zorgt dat zenuwprikkels sneller door het zenuwstelsel kunnen reizen.

a) negatieve signalen, die groei belemmeren en b) te weinig positieve signalen voor uitgroei van OPCs. Het is belangrijk te achterhalen uit welke signaalmoleculen deze obstakels bestaan, zodat we kunnen proberen therapieën te ontwikkelen die extra positieve signalen ontwikkelen en/of de negatieve signalen blokkeren. En op die manier genezing van myeline bevorderen. Enkele negatieve signaalmoleculen voor OPC uitgroei zijn al geïdentificeerd in MS. In dit proefschrift werd de rol van het eiwit fibronectine onderzocht.

Fibronectine is een belangrijk eiwit van de extracellulaire matrix; een netwerk van signaaleiwitten rondom cellen. In hoofdstuk 2 laten wij zien dat fibronectine nauwelijks wordt gemaakt in het gezonde zenuwstelsel, maar dat het zich ophoopt na beschadiging van myeline (demyelinisering, dus) in MS. Ook in diermodellen, waarin demyelinisering experimenteel wordt toegebracht, vinden we plaatselijk nieuw fibronectine. Belangrijke celtypen die fibronectine uitscheiden zijn astrocyten, microglia/macrofagen en bloedvatwand cellen (endotheel cellen) en daarnaast 'lekt' fibronectine waarschijnlijk uit het plasma van beschadigde bloedvaten. In sommige diermodellen geneest myeline spontaan en in dergelijke modellen zien we dat fibronectine wordt afgebroken tijdens het herstelproces. Echter, wanneer myeline niet geneest, zoals in een ander model en in MS, dan blijft ook fibronectine aanwezig. In dergelijke laesies met beperkt myeline herstel verandert ook de eiwitstructuur van fibronectine; dan klontert fibronectine samen tot aggregaten, waarschijnlijk onder invloed van weefselontsteking. Dus: de blijvende aanwezigheid van fibronectine aggregaten hangt samen met het falen van myeline genezing. Dat is een interessante correlatie, maar betekent het ook een causaal verband? Om dit te onderzoeken zetten we astrocyten in een kweekschaal aan tot het maken van fibronectine aggregaten. Vervolgens brachten we deze fibronectine aggregaten aan in experimentele, gedemyeliniseerde laesies, die normaal gesproken spontaan genezen. Echter, na toedienen van fibronectine aggregaten is het herstelproces beperkt. Daarom dragen fibronectine aggregaten in MS laesies bij aan het falen van myeline genezing.

Hoewel fibronectine aggregaten dus nadelig zijn voor myeline herstel, heeft de tijdelijke, niet-geaggregeerde fibronectine matrix, die wordt gemaakt na myeline beschadiging, waarschijnlijk eerst een gunstige invloed op OPCs. Maar hoe belangrijk is fibronectine voor OPCs? Misschien kunnen OPCs ook wel zonder fibronectine. Als dat het geval is, kunnen we misschien al in een vroeg stadium fibronectine verwijderen om aggregatie te voorkomen. Om dit te onderzoeken maakten we in hoofdstuk 3 gebruik van de conditionele knockout techniek. Door genetische manipulatie verkregen we muizen die verschillende typen fibronectine niet meer maken, namelijk: a) geen fibronectine in het plasma (bloed; plasma fibronectine), b) geen fibronectine gemaakt door astrocyten, c) geen fibronectine in plasma én niet van astrocyten. Daarna beschadigden we plaatselijk het myeline in het ruggenmerg (we maakten een demyeliniserende laesie), die spontaan zou moeten genezen in ongeveer drie weken. Inderdaad genazen alle laesies ook zonder de verschillende typen fibronectine. Echter, zonder fibronectine van astrocyten (astrocyt fibronectine) waren er minder OPCs. OPCs vermenigvuldigden zich minder goed zonder astrocyt fibronectine, en misschien werden ze ook minder goed aangetrokken. Inderdaad is uit eerdere studies bekend dat fibronectine de vermenigvuldiging van OPCs in een kweekschaal (*in vitro*) bevordert. De eiwitstructuur van astrocyt fibronectine verschilt van die van plasma fibronectine, want astrocyt fibronectine kan enkele extra domeinen bevatten: EIIIA en EIIIB. Geven deze extra domeinen misschien de gunstige signalen af van astrocyt fibronectine? Dit onderzochten we in een kweekschaal, en zo vonden we dat met name EIIIA, en niet EIIIB, van astrocyt fibronectine belangrijk is voor de vermenigvuldiging van OPCs. Fibronectine, vooral als het is gemaakt door astrocyten, heeft dus een positieve invloed op de vermenigvuldiging van OPCs, maar waarschijnlijk is fibronectine geen noodzakelijk signaal voor myeline genezing. Dit is een belangrijk inzicht bij het ontwikkelen van therapieën die het schadelijke aggregeren van fibronectine tegengaan.

Fibronectine kan niet alleen signalen geven aan OPCs, maar ook aan andere cellen die een rol spelen in MS en aanwezig zijn in de laesies. Een belangrijk voorbeeld hiervan wordt gevormd door microglia cellen, die binnen het zenuwstelsel een functie vervullen in ontstekingsreacties, en macrofagen, ontstekingscellen die uit het bloed infiltreren in het zenuwstelsel. Daarom onderzochten we in hoofdstuk 4 of fibronectine *aggregaten* de ontstekingseigenschappen van microglia en macrofagen beïnvloeden, hetgeen schadelijk zou kunnen zijn voor myeline genezing in MS. Uit deze studies bleek dat het uiterlijk van microglia en macrofagen verandert door fibronectine aggregaten; ze worden meer opgezwollen ('amoeboid'), een uiterlijk dat past bij ontstekingsactivatie. Ook worden macrofagen door fibronectine aggregaten aangezet tot het 'eten' van materialen uit hun omgeving ('fagocytose'). Zowel microglia als macrofagen kunnen specifieke ontstekingsprofielen aannemen, die passen bij bepaalde typen van weefselontsteking. In reactie op fibronectine aggregaten gebeurt dit echter waarschijnlijk niet. Interessant genoeg reageren microglia en macrofagen wel op fibronectine aggregaten door meer nitriet oxide uit te scheiden, een stofje dat betrokken is bij ontsteking in MS. Fibronectine aggregaten bevorderen dus bepaalde ontstekingseigenschappen van microglia en macrofagen. Hoe deze eigenschappen myeline

genezing beïnvloeden blijft vooralsnog onopgehelderd.

Eerder in deze samenvatting vergeleek ik verschillende typen van weefselgenezing met elkaar – de staart van de salamander, een wondje in uw vinger en myeline schade in MS. Inderdaad kennen deze processen gedeeltelijk overeenkomsten, ook al spelen ze zich af in verschillende weefsels. Bij het bestuderen van genezing in een bepaald weefsel, zoals van myeline in MS, kunnen we daarom leren van kennis die is opgedaan over genezing van ander weefsel, bijvoorbeeld een wond van de huid. Daarom werd in hoofdstuk 5, in een literatuur overzicht (*review*), vergeleken hoe fibronectine zich gedraagt na schade aan de huid (wonden), kraakbeen (artrose) en myeline (MS). Deze vergelijking maakte opnieuw duidelijk dat het ontstaan van fibronectine een gebruikelijke reactie is van het lichaam na weefselschade. Fibronectine heeft een positieve invloed op weefselgenezing, onder andere doordat het nieuwe cellen aantrekt en deze cellen helpt zich te vermenigvuldigen. Maar in een later stadium van weefselherstel, als er genoeg cellen zijn en deze overgaan tot het maken van nieuw weefsel, dan wordt fibronectine weer afgebroken. Afbraak van fibronectine correleert aan genezing van myeline, zoals onder andere aangetoond in hoofdstuk 2, maar ook aan genezing van de huid en kraakbeen. Echter, als de afbraak van fibronectine niet afdoende is, dan draagt dit ook in andere weefsels bij aan weefselschade. Fibronectine aggregaten verhinderen myeline genezing in MS (hoofdstuk 2), maar ook in chronische wonden en artrose verandert fibronectine van structuur; dan ontstaan fibronectine fragmenten. Met name in de context van artrose is veel onderzoek gedaan naar de schadelijke effecten van fibronectine fragmenten, die nieuwe kraakbeencellen remmen en daarnaast ook ongunstige ontstekingseigenschappen van lokale ontstekingscellen bevorderen. Fibronectine laat zich dus beschouwen als een steiger bij het aanmaken van nieuw weefsel: een handig eiwit, dat tijdelijk goed van pas komt, maar wel op tijd moet worden afgebroken om de wederopbouw van weefsel af te maken.

Samenvattend beschrijft dit proefschrift meerdere rollen van fibronectine tijdens myeline genezing in MS. Terwijl fibronectine aanvankelijke gunstige effecten heeft, met name door vermenigvuldiging van OPCs te stimuleren, is de blijvende aanwezigheid van fibronectine aggregaten in MS laesies ongunstig voor myeline genezing. Therapeutische interventies om de schadelijke invloed van fibronectine aggregaten te overwinnen zullen daarom waarschijnlijk bijdragen aan herstel van myeline, en daarmee herstel van zenuwstelsel functies, in MS. Dergelijke therapeutische interventies moeten nog worden ontwikkeld. Met name het blokkeren van de signalen die worden afgegeven door fibronectine aggregaten lijkt theoretisch interessant (besproken in hoofdstuk 6). Door het gebruik van dergelijke strategieën groeit de hoop dat myeline van MS patiënten in de toekomst zal kunnen teruggroeien, zoals de staart van de salamander.

Acknowledgements

Rather than scientific communications, all pages of this book could alternatively convey stories about the process of scientific discovery that was undertaken in pursuit of this thesis. Such stories of endurance and good fortune – illustrating the benefits of a Sunday morning discussion in the lab, but also highlighting the utter frustration of yet another past-dinner-time attempt to persuade a shRNA experiment to work (it never did) – would perhaps not proof anything, but that does not mean they would not be worth telling. Such stories could uncover a particular reality – a reality described by Virginia Woolf in '*A room of one's own*' as: 'something very erratic, very undependable – now to be found in a dusty road, now in a scrap of newspaper in the street, now a daffodil in the sun. It lights up a group in a room and stamps a casual saying. (…) But whatever it touches, it fixes and makes permanent.' In my stories of scientific activity and PhD thesis writing – a journey at times 'very erratic, very undependable' indeed –, I would always meet with enthusiastic and supportive colleagues and friends, whom I would like to 'fix' here and 'make permanent' in acknowledgement.

Before I identify the colleagues who have particularly supported me, let me stress that this PhD study could not have been undertaken without the Junior Scientific Masterclass MD/PhD program of the University Medical Centre Groningen and I am grateful that I was allowed to follow this program. Michiel Hooiveld provided valuable advice before I subscribed. Additional sources of financial support were: Marco Polo Fonds, Prinses Beatrix Fonds, J.K. de Cock Stichting and Groninger Universiteitsfonds, that supported my first visit to the Franklin laboratory in Cambridge. Further, the Research School of Behavioral and Cognitive Neuroscience from the University of Groningen and the Dutch Stichting MS Research also enabled my other visits to Cambridge. In addition, the Research School of Behavioral and Cognitive Neuroscience supported my attendance at two conferences, the European Meeting on Glial Cells in Health and Disease of 2009 (Paris) and of 2011 (Prague). In addition, I participated in the Brain Repair Centre Spring School of 2010 in Cambridge, which much benefitted my understanding of the field. Also, I was given the opportunity to take several useful courses at the Research School of Behavioral and Cognitive Neuroscience, in particular the courses on Laboratory Animal Science, Project Management and Statistics. Furthermore, the Dutch 'Stichting MS Research' hosted my attendance at the Dutch Multiple Sclerosis meetings of 2009 (Groningen), 2010 (Alphen aan den Rijn) and 2012 (Nijmegen), for which I am grateful. Finally, this thesis could not have been completed without the approval of Professor H.E. de Vries, Professor H.W.G.M. Boddeke and Professor H.P.H. Kremer, whom I would like to thank for doing so.

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This thesis is dedicated to Joshua van Beekum, who had to endure, more than anyone else, the personal disadvantages of this scientific journey, yet always committed, always believed, always loved.

Haarlem, February 2014
Josephine M.J. Stoffels

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List of abbreviations

ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AGG	aggregate
Aldh1L1	aldehyde dehydrogenase 1 family member L1
ANOVA	analysis of variance
APC	antigen-presenting cells
Arg	arginase
ATP	adenosine triphosphate
BBB	blood-brain barrier
BDNF	brain derived neurotrophic factor
BMM	bone-marrow macrophage
BMP	bone morphogenetic protein
BrdU	5-bromo-2′-deoxyuridine
BSA	bovine serum albumin
caMS	chronic active multiple sclerosis
CCL	chemokine ligand
cFn	cellular fibronectin
ciMS	chronic inactive multiple sclerosis
cKO	conditional knockout
CNP	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CNS	central nervous system
cr-EAE	chronic experimental autoimmune encephalomyelitis
CSF	cerebrospinal fluid
CSPG	chondroitin sulfate proteoglycan
CTRL	control
CWM	control white matter
CXCL	cysteine-x-cysteine motif ligand
DAPI	4′,6-diamidino-2-phenylindole
DIG	digoxigenin
DMEM	Dulbecco's modified Eagle medium
cDNA	copy-deoxyribonucleic acid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOC	deoxycholate
DPL	days post lesion
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EDA	extra domain A
EDB	extra domain B
EGF	epidermal growth factor
ER	endoplasmatic reticulum
ErbB	epidermal growth factor
FCS	fetal calf serum
Fn	fibronectin
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GalC	galactosylceramide
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GPR	G-protein coupled receptor

HDAC	histone deacetylase
HLA	human leukocyte antigen
HMBS	synthase
HMW	high molecular weight
Iba	ionized calcium-binding adapter molecule
IB4	isolectin-B4
Id	Inhibitor of DNA-binding
IFA	incomplete Freund's adjuvant
IFN	interferon
IGF	insulin-like growth factor
IHC	immunohistochemistry
IL	interleukin
iPSC	induced pluripotent stem cell
LDH	lactate dehydrogenase
Ln	laminin
LIF	leukemia inhibitory factor
LPS	lipopolysacharide
LXR	liver X receptor
MAC	macrophage
MAG	myelin associated glycoprotein
MAL	myelin and lymphocyte protein
MAPK	mitogen-acitvated protein kinase
MBP	myelin basic protein
M-CSF	macrophage colony stimulating factor
MCT	monocarboxylic acid transporter
MHC	major histocompatibility class
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MRF	myelin-gene regulatory factor
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	multiple sclerose
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate
NDS	normal donkey serum
NF	neurofascin
NG	neuron-glia chondroitin sulphate proteoglycan
NGF	nerve growth factor
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NLR	nucleotidebinding oligomerization domain receptor
NS	not significant
NT	neurotrophin
OA	osteoarthritis
OLG	oligodendrocyte
OPC	oligodendrocyte progenitor cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF-AA	platelet-derived growth factor-α

PDGFR-α	platelet-derived growth factor receptor-α
PFA	paraformaldehyde
pFn	plasma fibronectin
PI3	phospoinositide-3-kinase
PLP	proteolipid protein
PNS	peripheral nervous system
PPAR	peroxisome proliferator activator protein
PSA-NCAM	polysialylated-neural cell adhesion molecule
qPCR	quantitative polymerase chain reaction
rMS	remyelinated multiple sclerosis
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
RXR	retinoid X receptor
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SGalC	sulfogalactosylceramide
Shh	sonic hedgehog
SIRT	sirtuin
SNARE	soluble NSF attachment protein receptor
TBS	tris-buffered saline
Tcf	transcription factor
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TLR	toll-like receptor
TMEV	Theiler's murine encephalomyelitis virus
TNF	tumor necrosis factor
TRITC	tetramethylrhodamine
Trk	tyrosine kinase receptor
TuJ1	neuron-specific class III beta-tubulin
US	unstimulated
VAMPS	vesicle-associated membrane proteins
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
Wnt	wingless-int
YY	Yin Yang